DOCTOR OF PHILOSOPHY

Inhibition of PLK1 by p53 and by PLK1-targeted drugs

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Inhibition of PLK1 by p53 and by PLK1-targeted drugs

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This thesis is dedicated to my parents.

For their endless Love, Care and Support.

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Abbreviations

Aa	amino acid
APC/C	Anaphase Promoting Complex/ Cyclosome
APS	Ammonium persulfate
ATM	Ataxia-Telangiectasia Mutated
ATP	Adenosine triphosphate
ATR	Ataxia-Telangiectasia and Rad3 Related
Bbp	base pair
BrdU	Bromodeoxyuridine
CAD	Caspase Activated Dnase
CBP	cAMP Response Element Binding (CREB) Binding Protein
CDE	Cell cycle-Dependent Element
CDK	Cyclin Dependent Kinase
ChIP	Chromatin Immuno-Precipitation
CHR	Cell cycle genes Homology Region
CLE	CHR like elements
C-terminus	Carboxyl terminus
DC	Detergent Compatible
DDR	DNA Damage Response
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid

DNA-PK	DNA-dependent protein kinase
DREAM	DP, RB-like, E2F and MuvB
DTT	Dithiothreitol
E.V	Empty Vector
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EMI1	Early Mitotic Inhibitor 1
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FISH	Fluorescence in Situ Hybridisation
FITC	Fluorescein isothiocyanate
FOR20	FOP-related protein of 20 kDa
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H	Hour
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HR	Homologous Recombination
HRP	Horseradish peroxidase
ICAD	Inhibitor of CAD (Caspase Activated DNase)
IF	Immuno-fluorescence
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	Kilo base

kDa	Kilo-Dalton
LB	Lysogeny Broth
M	Molar
MDM2	Murine Double Minute 2
min	Minutes
MTOC	microtubule-organizing centre
Mut	Mutant
NF-Y	Nuclear Transcription Factor Y
NHEJ	Non-Homologous End Joining
Nlp	Ninein-like protein
NLS	Nuclear Localisation Signal
no.	Number
N-terminus	Amino-terminus
Orc2	Origin recognition complex 2
P	Phosphor
PAGE	Polyacrylamide gel electrophoresis
PBD	Polo Box Domain
PBIP1	Polo-Box Interacting Protein 1
PBS	Phosphate-buffered saline
PCAF	P300/CBP-Associated Factor
PCR	Polymerase Chain Reaction
PICH	PLK1-Interacting Checkpoint Helicase
PIKK	Phosphatidylinositol-3-Kinase-like Kinase

PLK	Polo-Like Kinase
PRR	Proline Rich Region
PTM	Post Translational Modification
RB	Retinoblastoma protein
RE	Responsive Element
RNA	Ribonucleic acid
SAC	Spindle Assembly Checkpoint
SDS	Sodium Dodecyl Sulfate
SP1	Specificity Protein 1
STLC	S-Trityl-L-cysteine
SV40	Simian Virus 40
TAD	Trans-activation Domain
TAE	Tris-acetate-EDTA
TAF	Transcription Activation Factor
TAFIID	Transcription Factor II D
TBP	TATA Binding Protein
TIF	Telomere dysfunction Induced Foci
Topors	topoisomerase I-binding protein
TSS	Transcription Start Site
UV	Ultraviolet
V	Volts
v/v	volume to volume
w/v	weight to volume

WB	Western Blotting
WT	Wild Type
°C	Degree Celsius
µ	Micro
53BP1	p53 Binding Protein 1

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Declaration

I hereby declare that I am the author of this thesis; that, unless otherwise stated, all references cited have been consulted by me; that the work of which this thesis is a record has been carried out by me, and it has not been previously accepted for a higher degree.

Maryam Ahmadi April 2018

I hereby declare that Maryam Ahmadi has completed the work presented in this thesis under my supervision. I confirm that she has fulfilled the conditions of the relevant Ordinance and Regulations of the University of Dundee, thereby qualifying her to submit this thesis in application for the degree of Doctor of Philosophy.

Doctor David W. Meek April 2018

Abstract

Polo like kinase 1 (PLK1) has many functions in the orchestration of cell division. It is an oncogene and its overexpression is associated with poor prognosis. PLK1 is a cell cycle-dependent protein; its levels are low in the early phases of the cell cycle and increase as cells go through the cycle, peaking in G2/M. In this thesis, our focus has been on two critical areas in PLK1 research.

1. Tumour suppressor p53 mediates transcriptional repression of *PLK1*. There have been several mechanisms suggested for this repression both by direct and indirect p53 involvement. In light of the recently proposed mechanism suggesting that p53-p21-DREAM-CDE/CHR is responsible for p53 mediated repression of G2/M proteins (including PLK1), we reassessed the event(s) by which p53 downregulates PLK1. While investigating the effect of different p53 stabilising agents on PLK1 levels, different agents were found to give rise to different cell cycle profiles. This could account for differences in the extent of PLK1 downregulation in response to different agents: i.e. that the levels of PLK1 after treatment reflect the phase in which the cycle is arrested. Further investigation showed that p53 mediated repression of PLK1 is partly p21 dependent, consistent with repression occurring (partly) through DREAM. Also, we found that different cells use different or overlapping mechanisms for this repression. Thus While in HCT116 cells, mutation of CDE/CHR elements (through which DREAM acts) abolished the PLK1 repression, U2OS cells were only partly dependent on CDE/CHR elements for this repression. Additionally, Serine 15 phosphorylation was found to be partly required for the repression and only wild type p53 (but not mutated forms of p53 that lack transactivation

capacity) could repress PLK1 expression. These data are consistent with the idea that p53 repression is indirect, but do not rule out other mechanisms.

2. PLK1 has been extensively studied as a target for cancer therapeutics. PLK1 inhibition causes arrest in prometaphase and activation of the DNA damage response. Part of this thesis aimed at investigating the DNA damage response (DDR) induced in mitosis by PLK1 inhibitor and the consequences of such inhibition. Also, the results were compared with the results obtained/reported in response to microtubule poison, nocodazole. Our investigations showed some differences in the mitotic arrest-induced DDR observed by PLK1 inhibition and microtubule poisons. PLK1 inhibition resulted in both telomeric and non-telomeric γ-H2AX foci which were not caspase/CAD dependent, whereas nocodazole treatment resulted in caspase dependent DNA damage which was mostly on telomeres. DNA damage sensing protein kinases involved in DDR by PLK1 inhibition were found to be ATR and DNA-PK, whereas for nocodazole DNA-PK was the main kinase involved. Also, in clonogenic survival assays, more surviving colonies were observed in response to PLK1 inhibitor as compared with nocodazole treatment. Interestingly, PLK1 inhibition resulted in recruitment of the 53BP1 (one of the components of the DNA repair pathway) in mitosis which may not be beneficial and suggests further investigations on chromosomal abnormalities that PLK1 inhibition may bring about.

Chapter 1: Introduction

1.1 Polo Like Kinases (PLKs)

Polo was the first identified PLK family member which was discovered in 1988 from Drosophila's genetic screens for mutants that could not undergo normal mitosis and meiosis (Sunkel & Glover, 1988). It is a serine/threonine kinase and is highly conserved between species from yeast to humans; each species having between one to several orthologues. However, in plants it has been reported that there are no PLKs (Karpov *et al.*, 2010). In Humans five homologues of polo have been identified to date (PLK1, PLK2, PLK3, PLK4 and PLK5).

PLKs are important regulators of the cell cycle progression and maintain DNA integrity. They orchestrate cell division through cooperation with Cyclin-dependent kinases (CDKs) (Strebhardt and Ullrich, 2006). The structures of all PLKs are similar to one another with two main features in their structure: **(a)** the amino terminal (N-terminal) kinase domain that can regulate the cellular functions of specific proteins by transferring phosphate groups from ATP to their serine/threonine amino acids, **(b)** a regulatory polo box domain which contains two (three in case of PLK4) signature motifs known as polo boxes at the carboxyl domain (C-terminal) which regulates the cellular localization of PLK1 and its substrate binding (Barr et al. 2004; Zitouni et al. 2014). The structure of PLK family proteins are depicted in Figure 1-1.

PLK1 is the most conserved member of the family and its functions in cell cycle progression have been better studied and understood. The Focus of this Thesis is on PLK1, but before going through PLK1, a brief introduction on other members of the family will be given.

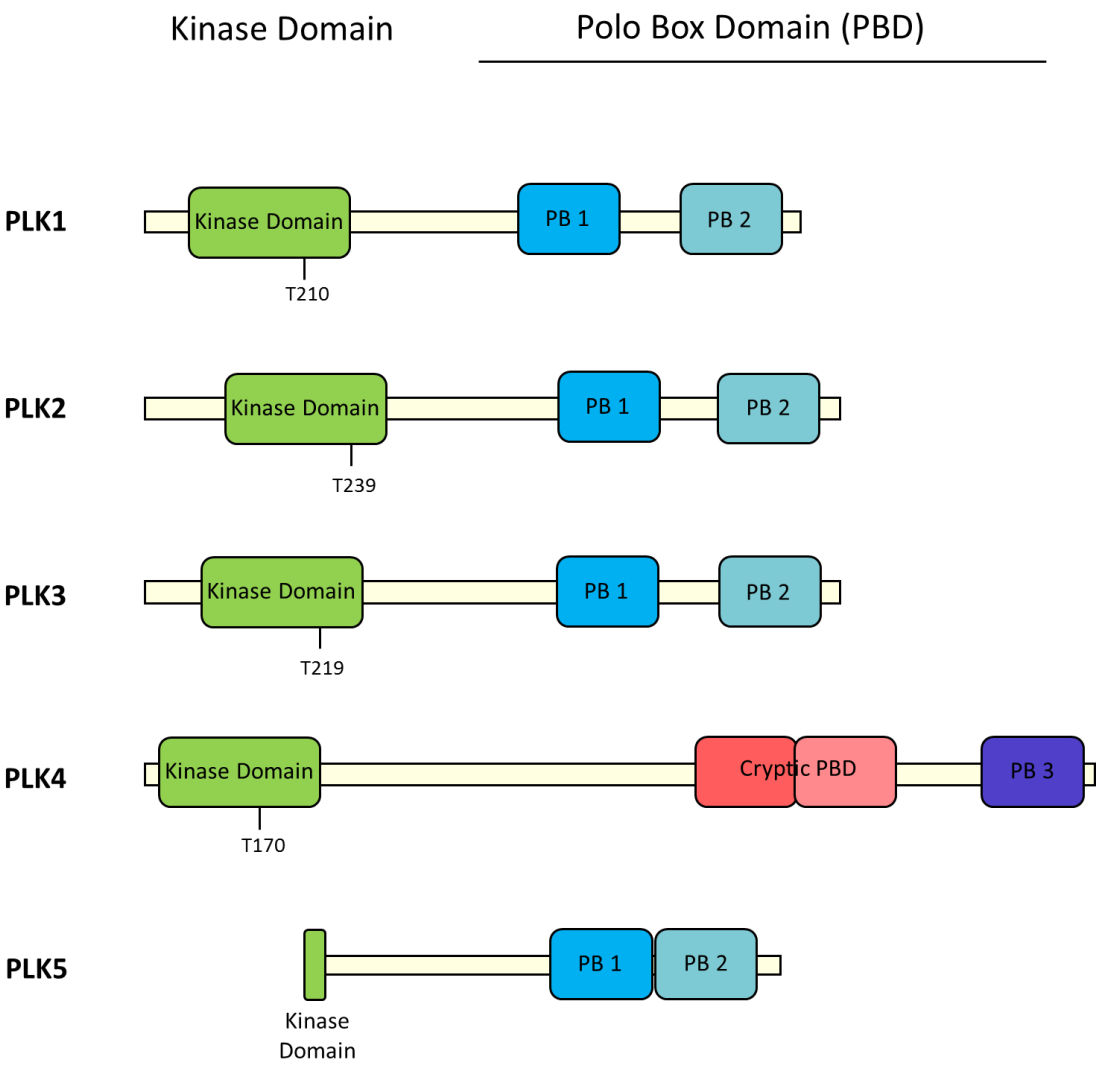


Figure 1-1. Schematic diagram of human PLK family structures.
Members of PLK family are depicted in the schematic. PLK1-4 all contain a protein kinase domain (green) in their N-termini. PLK1 activity is enhanced by phosphorylation at threonine 210 (T210) in the activating T loop, in the kinase domain. The residues equivalent to threonine 210 are conserved in PLK2, PLK3 and PLK4, as depicted. PLK5 contains the final fragment of the kinase domain only, due to an in-frame stop codon in exon 6 which is followed by a conserved ATG in the boundary of exons 6 and 7. PLK1, 2, 3 and 5, contain two polo boxes (PBD1 and PBD2, blue). PLK4 contain a cryptic polo box (orange) and a C-terminal Polo box (blue).

1.1.1 PLK5

PLK5 is the most recently identified PLK family and therefore not much research has been done on it (Andrysik *et al.*, 2010). PLK5 can be found in vertebrates only. Interestingly, human PLK5 lacks the catalytic kinase domain in the N terminus because of a stop codon in exon 6 which is followed by a conserved ATG in the boundary of exons 6 and 7. This results in expression of a short protein with final fragment of the kinase domain, the linker region and polo box 1 and 2. PLK5 has been reported to be expressed in the eye, brain and ovary of mice (de Carcer *et al.*, 2011). PLK5 expression is seen in nucleoli and can be induced by DNA damaging agents. The stress response of PLK5 has been reported to be independent of p53 (in contrast to PLK2 and PLK3). PLK5 downregulation has been reported in many brain tumours (de Carcer *et al.*, 2011) and its overexpression results in G1 arrest, DNA synthesis reduction and apoptosis (Andrysik *et al.*, 2010). This suggests its tumour suppressive activity.

1.1.2 PLK4

PLK4 (SNK/PLK-akin kinase) seems to be the most structurally divergent Polo family member. In the structure of PLK4, there is a cryptic polo box domain consisting of two tandem polo box domains (PBD1 and PBD2) and a third polo box (PB3) (Slevin *et al.*, 2012). PLK4 is a cell cycle dependent protein and its expression is low in G1 and gradually increases in S phase through M phase (peaks in M) (Fode *et al.*, 1994). PLK4 is highly expressed in high proliferative tissues and has been shown to be critical in mouse embryonic development (Swallow *et al.*, 2005). The best characterised function of PLK4 is in centriole

duplication during S phase (Habedanck *et al.*, 2005). PLK4 has been shown to be downregulated by p53, however despite the fact that there are three p53 binding sites in the promoter region of PLK4, p53 does not bind to these sites, suggesting an indirect repression mechanism (Li *et al.*, 2005).

1.1.3 PLK3

PLK3 (Proliferation-related kinase, PRK) was first characterised as a fibroblast growth factor 1 (FGF1)-induced immediate early gene (Donohue *et al.*, 1995). It is believed to be a stress induced tumour suppressor; in response to DNA damage PLK3 activity increases rapidly in an ATM dependent manner (Helmke, Becker and Strebhardt, 2016).

PLK3 is a p53 target gene and upon irradiation its transcription is activated by p53 through a p53 responsive element (p53RE) in PLK3 promoter (Jen and Cheung, 2005). PLK3 interacts with p53 and phosphorylates it on serine 20 leading to p53 stabilisation and checkpoint activation. This finding suggests that PLK3 is a tumour suppressor and functions in regulating cell proliferation (Xie *et al.*, 2001).

The expression of PLK3 and its cell cycle dependency remain unclear as different laboratories reported differently (Bahassi *et al.*, 2002; Zimmerman and Erikson, 2007; Chase *et al.*, 1998; Ouyang *et al.*, 1997). PLK3 has been reported to be downregulated in some cancers including lung, head and neck, osteosarcoma (Li *et al.*, 1996; Dai *et al.*, 2000; Lv *et al.*, 2015).

1.1.4 PLK2

Like PLK5, PLK2 is found in vertebrates only. PLK2 (Serum inducible kinase, Snk) is believed to be a tumour suppressor as it is downregulated in some cancers and its ectopic expression results in apoptosis (Syed *et al.*, 2006). PLK2 has been reported as a transcriptional target of p53, with three p53 binding sites in the promoter region of its gene; two sites for p53 mediated transcriptional activation and one for repression (Burns *et al.*, 2003). PLK2 has also been reported to be induced, in a p53 dependent manner, following irradiation and activation of the G2/M checkpoint, suggesting that it is a stress response gene (Shimizu-Yoshida *et al.*, 2001; Burns *et al.*, 2003). PLK2 has been suggested to have a role in centriole duplication which is dependent on PLK4 (Cizmecioglu *et al.*, 2008).

1.2 PLK1

PLK1 is the first discovered and best characterised of PLK family. It was first identified in 1994 and the founders/authors reported it being maximally expressed in tissues with a high mitotic index suggesting its function in cell proliferation (Golsteyn *et al.*, 1994). PLK1 consists of 603 amino acids and its molecular mass is approximately 68 kDa. PLK1 expression, activity and localisation need to be tightly regulated throughout the cell cycle to ensure chromosome stability/integrity (Liu, Sun and Wang, 2017).

1.2.1 PLK1 structure and localization

As mentioned before, all members of the PLK family, including PLK1, contain 2 functional domains; N terminal kinase domain and C terminal polo box domain (comprises 2 polo boxes which function as a single unit). Both of these domains are required for PLK1 activity. Phosphorylation of threonine 210 (T210) in the T loop of PLK1 by Aurora A enhances the catalytic activity of PLK1. This phosphorylation induces a stable conformational change which enables PLK1 interaction with other molecules (Lowery, Lim and Yaffe, 2005). Activated PLK1 can then localise to the specific sites (phospho-serine/threonine containing peptides) through its PBD which is a phosphopeptide (phospho serine or phospho threonine) binding domain (Elia, Cantley and Yaffe, 2003). Interestingly deletion of PBD of PLK1 resulted in substantial increase in its catalytic activity suggesting a role for PBD in negatively regulating PLK1 activity (Mundt *et al.*, 1997).

Binding of PLK1 PBD to proteins that have already been phosphorylated and marked as a docking site for the PBD, shows that priming phosphorylation of the substrate is crucial for the PLK1 localisation and functions (Elia, Cantley and Yaffe, 2003). The priming phosphorylation/generation of the phosphoepitope happens either by self priming (by PLK1) or by a pro-directed kinase (such as CDK1) (Lee *et al.*, 2008). After localisation of PLK1 by PBD, PLK1 kinase domain can perform its functions by phosphorylating its substrates.

PLK1 localization is highly dynamic and differs in each step of the cell cycle. During interphase and prophase PLK1 is localised at centrosomes but then in prometaphase and metaphase it moves to the kinetochores and spindle poles to regulate assembly of the kinetochores and contribute to the spindle assembly

checkpoint (Degenhardt and Lampkin, 2010). It then translocate to the central spindles and midzone/midbody in anaphase and telophase (Zitouni *et al.*, 2014).

1.2.2 PLK1 functions

PLK1 is a major kinase in mitosis and functions almost in every step during mitosis. It has roles in mitotic entry, centrosome maturation, bipolar spindle formation, chromosome segregation and mitotic exit/cytokinesis. Below is a brief description of main functions of PLK1.

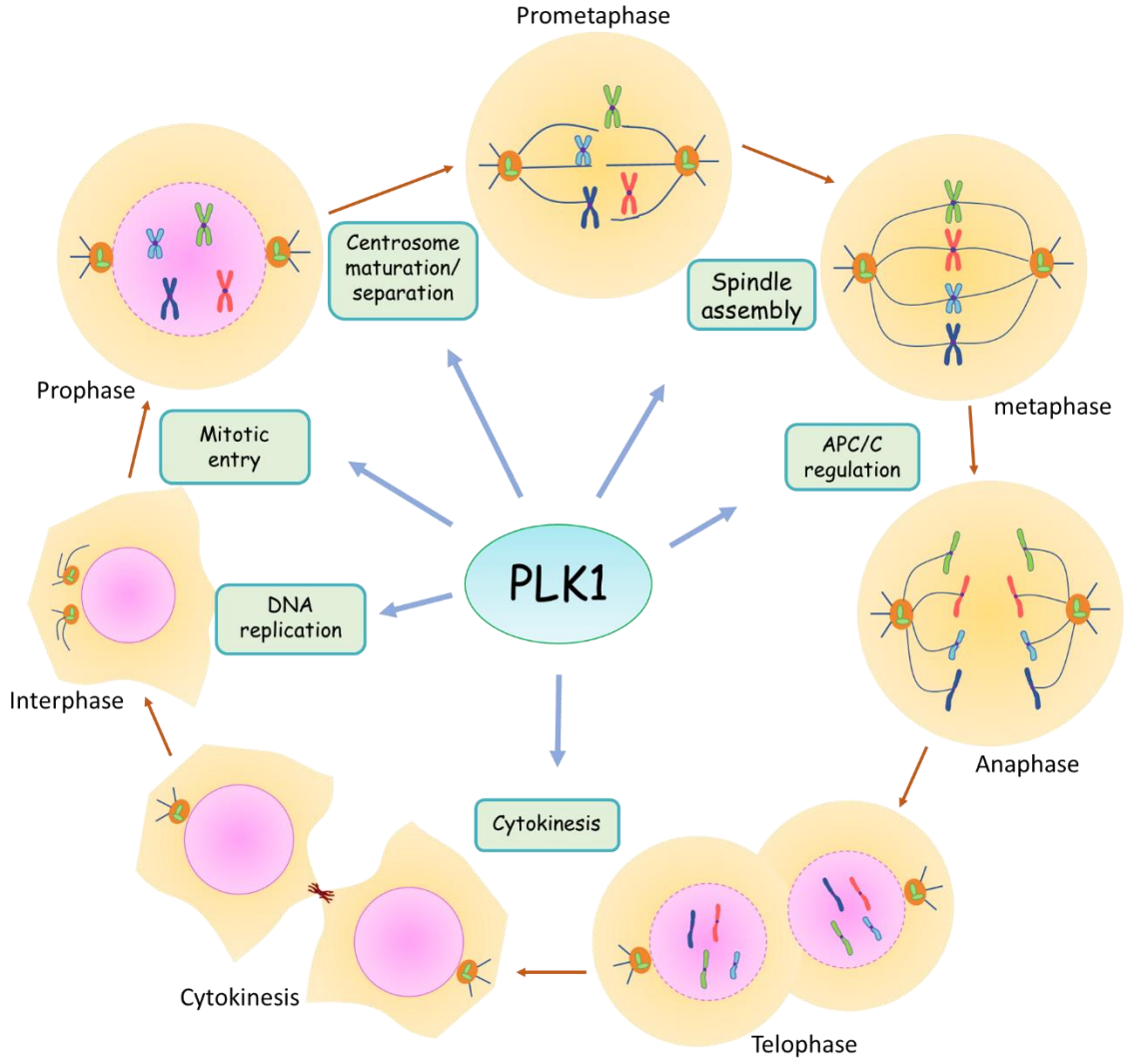


Figure 1-2. PLK1 functions throughout the cell cycle.
PLK1 has many roles during the cell cycle. The most well studied functions of PLK1 are in mitosis. PLK1 has been shown to be involved in various aspects of mitotic progression such as mitotic entry, centrosome maturation and separation, spindle assembly, APC/C regulation and cytokinesis. Non-mitotic roles for PLK1 have also been suggested. In interphase, PLK1 has been reported to be involved in DNA replication.

The first main function of PLK1 in mitosis is to promote **mitotic entry**. The key to entry into mitosis is the activation of CDK1-Cyclin B. CDK1 is phosphorylated and inhibited by WEE1 and MYT1 protein kinases and de-phosphorylated and activated by CDC25C phosphatase. In order to promote mitotic entry, PLK1 activates CDK1-Cyclin B1 by inhibiting WEE1 and MYT1 and activating CDC25C (Toyoshima-Morimoto *et al.,* 2001; Van Vugt, Brás and Medema, 2004; Nakojima *et al.*, 2003). There's also evidence that PLK1 can promote mitotic entry by direct phosphorylation of Cyclin B1 at centrosomes (Toyoshima-Morimoto *et al.*, 2001)

In response to DNA damage, PLK1 is inactivated through phosphorylation by ATM and cell cycle halts (Van Vugt and Medema, 2005). In order to resume cell cycle progression (this is called recovery), activity of PLK1 is required. Synergistic cooperation of Aurora kinase A and its co-factor Bora, results in direct phosphorylation of PLK1 at threonine 210 by Aurora A (occurs several hours prior to mitotic entry). This enhances the catalytic activity of PLK1 which then results in CDK1 activation and mitotic entry (Macůrek *et al.*, 2008; Seki *et al.*, 2008). Even though PLK1 functions in activation of CDK1-Cyclin B1 complex, it appears that its activity is not essential for entry into mitosis as using an inhibitor of PLK1 does not block mitotic entry but instead results in mitotic arrest (Steegmaier *et al.*, 2007).

The next role of PLK1 is **centrosome maturation**. Centrosomes are the microtubule-organizing centre (MTOC) in animal cells. They consist of two centrioles and the surrounding pericentriolar material (dense mass of proteins such as γ-tubulin, pericentrin and ninein, which are involved in microtubule nucleation and anchoring) (Schatten, 2008). Centrosomes duplicate during S

phase and their maturation occurs in preparation for mitosis. In this process accumulation of γ-tubulin ring complexes and recruitment of other components leads to morphological and biochemical changes in pericentriolar material (Khodjakov and Rieder, 1999). These changes enhance the microtubule nucleation potential.

PLK1, has been suggested to phosphorylate pericentrin, an initiating step in centrosome maturation which results in recruitment of γ-tubulin and other pericentriolar material proteins (Sunkel and Glover, 1988; Lane and Nigg, 1996; Nigg, Blangy and Lane, 1996; Lee and Rhee, 2011) . PLK1 also phosphorylates a centrosomal protein called Nlp (ninein-like protein) which is involved in microtubule anchoring and nucleation. During interphase, Nlp interacts with γ-tubulin ring complexes to assist in microtubule organisation. During mitosis, however, phosphorylation of Nlp leads to displacement of Nlp from centrosomes. This replacement has been proposed to be a prerequisite for the recruitment of other proteins (still unidentified), which then results in increased microtubule nucleation potential (Casenghi *et al.*, 2003).

PLK1 also has roles in **centrosome separation** which is required for the formation of bipolar spindles. PLK1 targets Eg5, a kinesin like motor protein also known as KIF11, which hydrolyses ATP to convert chemical energy into mechanical work (Zima, 1998). PLK1 in conjunction with CDK1 phosphorylates NEK9 in prophase. Activated NEK9 then phosphorylates and activates NEK6/7, leading to Eg5 phosphorylation and its localisation/recruitment to the centrosomes (Bertran *et al.*, 2011).

PLK1 depletion has been shown to reduce the number of attached microtubules to the kinetochores. Kinetochores are protein structures at the centromere of

chromatids at which spindle microtubules attach. As mentioned before, PLK1 localises to the kinetochores in prometaphase, which further indicates that it may function at these cellular elements to perform its role in **stabilising microtubule-kinetochore attachments** (Sumara *et al.*, 2004). PLK1 recruitment to the kinetochores has been reported to be dependent on a protein at the centromere called PBIP (polo box interacting protein 1) (Kang *et al.*, 2006). PLK1 uses a self-priming mechanism and phosphorylates PBIP1 to create a binding site for PBD and dock itself to centromere (Kang *et al.*, 2006). However, in early mitosis, PLK1 causes delocalization and degradation of BPIP1 from kinetochores (Kang *et al.*, 2006), whereas PLK1 continues to be localised at kinetochores until telophase. This shows that there should be other proteins contributing to PLK1 localisation at kinetochores. BubR1 is one of the PLK1 substrates whose phosphorylation by PLK1 has been suggested to be important for stable microtubule-kinetochore attachments. Elowe et Al. showed that BuBR1 phosphorylation by PLK1 at unattached kinetochores which are not under tension is higher than attached kinetochores. This phosphorylation continues until metaphase when the tension has established between sister chromatids (Elowe *et al.*, 2007).

As PLK1 levels are particularly higher in the kinetochores of unattached chromosomes (which are not under tension) (Ahonen *et al.*, 2005) and because PLK1 phosphorylates substrates required for spindle assembly checkpoint (SAC) activation such as BubR1, Bub1 and PICH (Qi, 2006, Suijkerbuijk *et al.*, 2012; Baumann *et al.*, 2007), it has been suggested that PLK1 might have roles in the SAC activation. However, PLK1 inhibition causes prolonged arrest in prometaphase as a result of SAC activation (as evidenced by bright and intense staining of SAC proteins, Bub1, BubR1 and Mad1) (Lénárt *et al.*, 2007). So, the

role of PLK1 in unattached kinetochores is perhaps in regard to promoting proper microtubule-kinetochore attachment and not related to regulation of SAC.

Localisation of PLK1 to the central spindles and midbody in late anaphase and telophase respectively suggested its role during late stages of the cell cycle. PLK1 initiates **mitotic exit** by inhibitory phosphorylation of Emi1 (early mitotic inhibitor 1) which leads to activation of E3 ubiquitin ligase APC/C (anaphase promoting complex/cyclosome). This results in cyclin B degradation by 26S proteasome and hence inactivation of CDK1 (Moshe *et al.*, 2004).

PLK1 also recruits RhoGEF Ect2 to the central spindles (during anaphase). This protein has a fundamental role in signalling from the midzone to the cortex which is a requirement for cytokinesis induction. This promotes the activation of RhoA GTPase which causes generation of cleavage furrow by assembling and regressing the contractile ring and initiating **cytokinesis** (Petronczki *et al.*, 2007).

In addition to its critical role in mitosis, PLK1 has been reported recently to have functions in **DNA replication during S phase**. An RNAi approach used by Yim and Erikson showed that PLK1 is required for DNA replication during cell cycle progression as depletion of PLK1 caused disruption of the pre-replicative complex formation at G1/S transition and reduction in DNA synthesis during S phase (Yim and Erikson, 2009). Also, PLK1 recruitment to the centrosomes by centrosomal protein FOR20 (FOP-related protein of 20 kDa) has been shown to be essential in DNA replication and S phase progression (Shen *et al.*, 2013). In another study, Orc2 (origin recognition complex 2), one of the subunits of pre-

replicative complex, has been reported as a target of PLK1 under stressed conditions. PLK1 phosphorylates Orc2 at serine 188. This phosphorylation is enhanced when DNA replication is under challenge. Cells that express an Orc2 mutant (S188A), failed to maintain the functional pre-replicative complex in response to DNA replication stress (Song *et al.*, 2011).

1.2.3 PLK1 and cancer

Consistent with its functions in cell division and proliferation, it is not surprising that PLK1 has been linked to both development and progression of cancers. PLK1 overexpression has been reported in many cancer types including non-small cell lung cancer (Wolf *et al.*, 1997), breast cancer (Wolff *et al.*, 2000), (Weichert *et al.*, 2005; A Ahr *et al.*, 2002), ovarian cancer (W Weichert *et al.*, 2004a), colorectal cancer (Takahashi *et al.*, 2003), glioblastoma (Dietzmann *et al.*, 2001), pancreatic cancer (Gray Jr. *et al.*, 2004), gastric carcinoma (Tokumitsu *et al.*, 1999), head and neck squamous cell carcinoma (Knecht *et al.*, 1999) , melanoma (Strebhardt *et al.*, 2000; Kneisel *et al.*, 2002), prostate cancer (Wilko Weichert *et al.*, 2004) and non-Hodgkin lymphoma (Mito *et al.*, 2005). PLK1 overexpression is a marker of poor prognosis and has been linked to higher tumour grades and increased risk of metastasis (Wolf *et al.*, 1997; Wilko Weichert *et al.*, 2004; W Weichert *et al.*, 2004; Kneisel et al., 2002; Ahr et al., 2002; Zhang, Zhang and Kong, 2013). A study conducted by King and colleagues showed an association between PLK1 overexpression and p53 mutation. Also, patients who had both PLK1 overexpression and p53 mutation showed worse survival as compared to patients having only PLK1 overexpression or p53 mutation (King *et al*., 2012*)*.

As *PLK1* is rarely mutated, it had been suggested that overexpression of PLK1 in cancer cells might be a consequence of cancer (and increased proliferation) and not the cause of it. But ectopic/constitutive expression of PLK1 in a normal mouse embryonic fibroblast line (NIH3T3) resulted in the malignant transformation of the cells and allowed them to grow in soft agar and caused tumours in nude mice, indicating its oncogenic potential (Smith *et al.*, 1997). In line with that, there has been evidence that rate of proliferation and cell cycle progression of cancer cells (but not normal cells) is highly suppressed by PLK1 depletion. This indicates that cancer cells are highly addicted to PLK1 and hence validating PLK1 as a potential therapeutic target (Liu, Lei and Erikson, 2006).

1.2.4 PLK1 small molecule inhibitors

PLK1 has been extensively studied as a target for cancer therapy as it is required for the survival and proliferation of the cells and is overexpressed in many cancer types (Lénárt et al. 2007, Rudolph et al. 2009). Overexpression of PLK1 has been reported to cause multi-nucleation (Mundt *et al.*, 1997). Expression of hyperactive PLK1 in cells that undergo DNA damage-induced G2 arrest causes recovery from G2 arrest (Van Vugt, Brás and Medema, 2004). Constitutive expression of PLK1 caused malignant transformation of mammalian cells (Smith *et al.*, 1997). Also, PLK1 phosphorylates tumour suppressor p53 to inhibit its transactivation activity and pro-apoptotic function (Ando *et al.*, 2004). All this, evidence, indicates that PLK1 is a strong target for therapeutic intervention for cancer.

Several PLK1 small molecule inhibitors have been manufactured and are in pre-clinical or clinical development. They are capable of inducing mitotic arrest and cause apoptosis in several cancers (Lénárt et al. 2007; Rudolph et al. 2009). As with any other anti-mitotic drug, PLK1 inhibitors maintain the spindle assembly checkpoint activated and prevent the cell cycle progression. PLK1 inhibition has shown promising results in different haematological and solid tumours (Strebhardt, 2010; Schöffski, 2009).

PLK1 inhibitors either target the kinase domain of PLK1 (ATP competitive inhibitors) or the PBD (non-ATP competitive/ substrate specific inhibitors). As the ATP binding pocket is a classic target for the design of kinase inhibitors, the first PLK1 inhibitors were those targeting the kinase domain. These inhibitors reduce PLK1 expression by interfering with its catalytic activity. BI2536 is an example of this class of inhibitors which we have used in this thesis (chapter 4). One drawback of such ATP competitive inhibitors is that the high conservation of ATP binding sites in different kinases provides challenges in identifying inhibitors with the appropriate specificity. Also, developing resistance to inhibitors that target the ATP binding site is not uncommon as there are chances of mutations in the kinase domain (Daub, Specht and Ullrich, 2004). So, in the light of making inhibitors that do not compete with the ATP and instead they compete for the PLK1 substrates, inhibitors that target the PBD were identified. PBD is specific to PLKs and hence a potential target to develop selective inhibitors. However, some of the PLK1 substrates do not require interaction with PBD to be recognised by PLK1 kinase domain for phosphorylation. This brings limitations into the development of inhibitors of PLK1 which target PBD (Park *et al.*, 2010).

BI2536: It is a potent, ATP competitive inhibitor of PLK1 with an IC50 value of 0.83 nM. It is well tolerated and acts with high specificity and selectivity (more than 1000 fold more selectivity for PLK1 compare to a large panel of other kinases) (Schöffski, 2009). However, it has effects on the activity of other PLKs such as PLK2 and PLK3 (which are potential tumour suppressors) with relative IC50s of 3.5 nM and 9 nM respectively. Nevertheless, since other PLK members are believed to have their roles in G1 and S phases, BI2536 is considered to be a specific inhibitor of PLK1 during mitotic phase (Lénárt et al. 2007). BI2536 leads to arrest in prometaphase, aberrant mitotic spindles and cell death in a large panel of cancer cell lines (Lénárt et al. 2007, Steegmaier et al. 2007). Moreover *in vivo* studies demonstrated a high efficacy at well-tolerated doses causing regression of human tumour xenografts in nude mice (Steegmaier *et al.*, 2007). It is now used in clinical studies of patients with advanced or metastatic cancers and has been reported to be well-tolerated with neutropenia as the main side effect (Strebhardt, 2010).

1.2.5 Cell cycle Regulation of PLK1 levels and enzymatic activity

PLK1 levels are tightly regulated throughout the cell cycle at mRNA and protein levels. Golsteyn and colleagues showed that PLK1 protein levels and distribution changes as cells go through the cell cycle and peak in G2/M. They also showed that tissues with high mitotic index show more PLK1 expression, consistent with its role in cell proliferation (Golsteyn *et al.*, 1994).

Various transcriptional repressors and activators are involved in early and late phases of the cell cycle respectively for *PLK1* transcriptional regulation. There

are elements/sequences in the promoters (close to transcription start site) of genes with high expression in late stages of the cell cycle such as *PLK1* and *CDC25C*. These elements are called CDE/CHR (cell cycle dependent element/cell cycle gene homology region) and are believed to be responsible for cell cycle dependency of these genes by negatively regulating them in early stages of the cell cycle. Deletion mutation in this region was shown to cause loss of cell cycle specific transcriptional regulation of PLK1 as evidenced by increased PLK1 level in G1 (Uchiumi, Longo and Ferris, 1997).

The mechanism by which PLK1 and other CDE/CHR contained promoters are regulated is through the action of **DREAM complex** (DP, RB-like, E2F and MuvB). It is composed of MuvB core complex (multi vulval class B; LIN9, LIN37, LIN52, LIN54 and RBBP4), E2F4-5, DP1 (dimerization partner 1) and pocket proteins p130 or p107 (related to the retinoblastoma tumour suppressor pRB) (Litovchick *et al.*, 2007). The repression mechanism is based on the hypophosphorylation state of RB-like pocket proteins p130/p107 in early phase of the cell cycle. In this state, p130/107 join other proteins and form the DREAM complex. Binding of DREAM to CDE/CHR elements represses the transcription (Mannefeld, Klassen and Gaubatz, 2009; Guiley et al., 2015; Litovchick et al., 2007) (**Figure 1-3**).

As the cells progress through the cell cycle, activated cyclin/CDK progressively phosphorylates p130/p107 leading to their deactivation and disassembly of DREAM complex (Guiley *et al.*, 2015). Additionally and importantly, the transcriptional repressors p130/107 and E2F are substituted with activators B-MYB and later FOXM1. MuvB association with B-MyB or FOXM1 switches the repressive DREAM complex to B-MYB-MuvB complex (MMB), FOXM1-MMB

complex or FOXM1-MuvB complex (Sadasivam, Duan and DeCaprio, 2012; Sadasivam and DeCaprio, 2013). This is a switch where the complex become an activator. This switch in protein binding to CDE/CHR is responsible for the transcriptional regulation of PLK1 during the cell cycle (**Figure 1-3**).

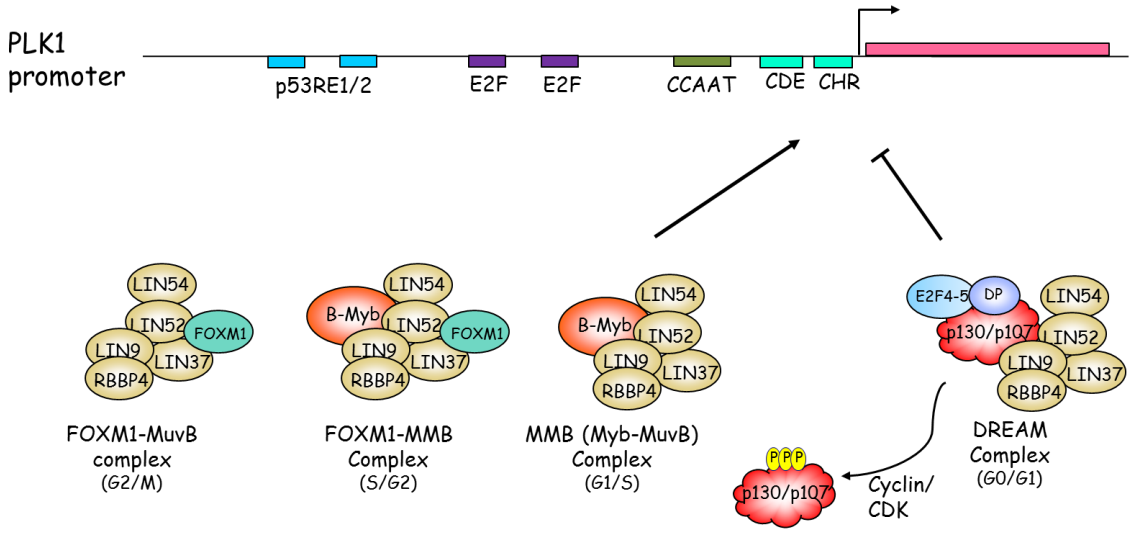


Figure 1-3. Schematic representation of PLK1 transcriptional regulation during the cell cycle.
The repression of PLK1 in G0/G1, is based on the hypo phosphorylation of RB-like pocket proteins p130/p107. Hypophosphorylation state of p130/107 allows them to join other proteins and form the inhibitory DREAM complex. As the cells progress through the cell cycle, activated cyclin/CDK progressively phosphorylate p130/p107 causing their deactivation and subsequently DREAM complex disassembly. Additionally, and importantly the transcriptional activators B-MYB and later FOXM1 displace the transcriptional repressors p130/107 and E2F. MuvB association with B-MyB or FOXM1 switches the DREAM complex to B-MYB-MuvB complex (MMB), FOXM1-MMB complex or FOXM1-MuvB complex which are activators.

Not only the levels of PLK1, but also its protein kinase activity is cell cycle regulated, which increases during G2/M transition and peaks in mitosis. Several hours prior to mitosis, CDK1 phosphorylates Bora (a cofactor of Aurora A). This promotes the PLK1-Bora interaction at PLK1 PBD which causes conformational changes to PLK1 structure. As a result Aurora A can phosphorylate PLK1 at its T loop (T210) and activate it (Macůrek *et al.*, 2008; Seki *et al.*, 2008).

PLK1 levels and activity decreases at the end of mitosis. CDH1 (APC/C activator) recognises and binds to PLK1 D-Box (destruction Box) and activates APC/C. Activated APC/C then targets PLK1 for degradation by 26S proteasome (Lindon and Pines, 2004).

1.2.6 PLK1 in DNA damage response

Cells are constantly undergoing endogenous or exogenous DNA damage which, if not repaired, could cause genetic instability. Cells undergoing DNA damage in interphase, particularly in S and G2 phase, cause activation of the G2 checkpoint and cell cycle arrest (Checkpoints will be explained in section 4.1.1) (Hyun, Hwan and Jang, 2014). PLK1 has been suggested to be a target for G2/M checkpoint in response to DNA damage as cells containing constitutively active mutants of PLK1 (T210D or S137D/T210D) can override the DNA damage activated checkpoint (Smits *et al.*, 2000).

In response to DNA damage ATM/ATR phosphorylate Bora at threonine 501. This phosphorylation subjects Bora for recognition by an E3 ubiquitin ligase (SCF-β-TRCP) and causes its degradation. As a result, PLK1 cannot be phosphorylated and activated by Aurora A (Qin *et al.*, 2013). After restoration of

DNA damage, PLK1 activity is required for checkpoint recovery and resetting the cell cycle (Macůrek *et al.*, 2008). One of the mechanisms involves PLK1 mediated regulation of a kinase protein called MFP (mitosis promoting factor). MPF consists of a catalytic subunit called cdc2 (also known as CDK1) and an activation subunit called cyclin B1 and stimulates mitotic entry (Nigg, 2001). PLK1 phosphorylates and activates CDC25c, which is an activator of cdc2, and also enhances the nuclear import of cyclin B1 eventually leading to mitotic entry (Gheghiani *et al.*, 2017).

Downregulation of PLK1 activity in response to DNA damage has been reported in mitosis too, where PLK1 is already activated. This mechanism is ATM/ATR dependent and involves dephosphorylation of PLK1 at threonine 210 by protein phosphatase 2A (PP2A) (Jang *et al.*, 2007).

DNA damage not only affects the activity of PLK1, it also represses PLK1 protein and mRNA levels in a p53 dependent manner (McKenzie *et al.*, 2010).

1.2.7 PLK1 and p53

PLK1 and p53 interact with each other in a negative feedback loop (**Figure 1-4**). In response to DNA damage, p53 has been reported to downregulate PLK1 by direct and indirect mechanisms. p53 has been shown to repress the *PLK1* gene expression by direct binding to the *PLK1* promoter at p53REs (McKenzie *et al.*, 2010). p53 has also been suggested to repress *PLK1* indirectly by inducing its downstream effector, p21. p21 inhibits cyclin/CDK and results in the formation of DREAM complex which is a repressor of *PLK1* (Fischer, Quaas, Nickel, *et al.*, 2015). p21 has also been shown to repress *PLK1* by disrupting the interaction

between cdk2 and NF-YA. NF-YA then binds to p21 and anchors it to CCAAT box resulting in *PLK1* transcriptional repression (Lin *et al.*, 2014). p53 has also roles in repression of FOXM1, an oncogenic transcription factor that acts in a positive feedback loop with PLK1 (Barsotti and Prives, 2009, Pandit, Halasi and Gartel, 2009). PLK1 repression has also been reported by p53 interaction with E2F1 to form an inhibitory p53–E2F1–DNA complex on the *PLK1* promoter leading to suppression of E2F1 mediated *PLK1* expression (Zhou *et al.*, 2013).

On the other hand, PLK1 also inhibits the function of p53 both directly and indirectly. Ando and colleagues showed that, PLK1 physically interacts with and phosphorylates p53. This results in loss of p53 transactivation activity and pro-apoptotic functions through inhibition of p53 mediated expression p21 and apoptosis inducing genes such as BAX (Ando *et al.*, 2004). PLK1 can also indirectly inactivate p53 by promoting its degradation. Phosphorylation of serine 260 of MDM2 by PLK1 may contribute to MDM2 stabilisation and binding to p53, thereby promoting p53 degradation (Dias *et al.*, 2009). Also, PLK1 by activation of CDC25C causes dephosphorylation of p53 at serine 15, which again increases the affinity of MDM2 to bind to p53 and promotes MDM2-mediated turnover of p53 (Chen *et al.*, 2006). PLK1 has been shown to phosphorylates Topors (topoisomerase I-binding protein) at serine 718, leading to enhancement of p53 ubiquitination and degradation (Yang *et al.*, 2009).

These evidences indicate the presence of a negative feedback loop between p53 and PLK1, with p53-mediated repression of *PLK1* transcription and PLK1-mediated inhibition of p53 function and promotion of its degradation. As the focus of chapter 3 of this thesis is the repression of *PLK1* by p53, the next sections will give us an overview of p53 for a better understanding.

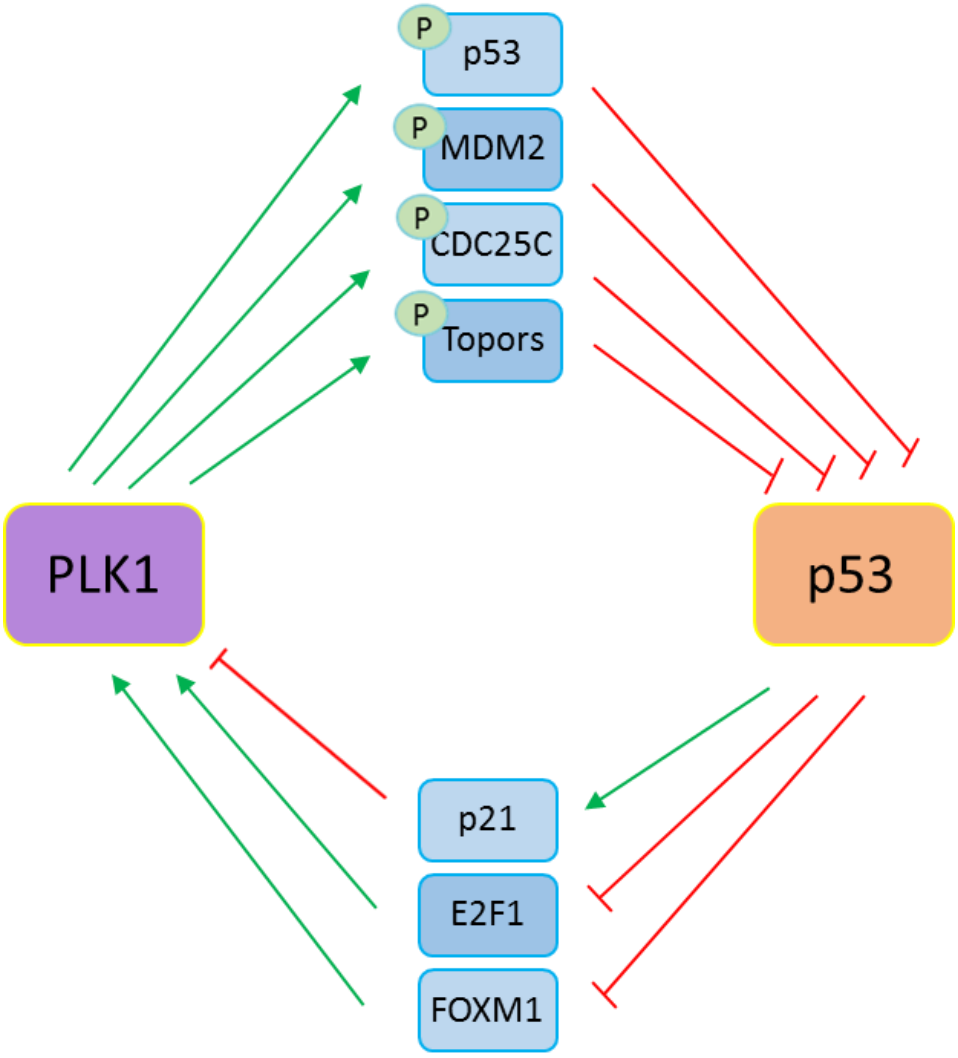


Figure 1-4. Negative feedback loop between PLK1 and p53.
There is a negative feedback loop between PLK1 and p53, with PLK1 mediating inhibition of p53 function and promoting its degradation and p53 mediating PLK1 repression. Different mechanisms, illustrated in the figure, have been explained in the text.

1.3 p53

p53 was first discovered in 1979 by several independent groups as a protein of approximately 53 KDa that was found bound to the Simian Virus 40 Large T-antigen (a known oncogene) (Lane and Crawford, 1979; Linzer and Levine, 1979; Kress *et al.*, 1979). p53 was then reported to be highly expressed in transformed cells compared to untransformed cells. However after irradiation, untransformed cells underwent a rapid increase in p53 levels which was attributed to post translational stabilization of p53 (Maltzman and Czyzyk, 1984). p53 was then found to be localised in the nucleus of the transformed fibroblast cells but in the cytoplasm of untransformed fibroblasts. Based on the above reports, the first decade of p53 research, pointed to p53 as an oncogene (Rotter, Abutbul and Ben-Ze'ev, 1983).

However, later it was discovered that the cDNAs used in initial experiments encoded mutant forms of p53 and although mutant p53 could cause transformation of cells, wild type p53 could suppress transformation (Finlay, Hinds and Levine, 1989). Since then, the main focus of research on p53 has been on its tumour suppressive role and it has been regarded as "guardian of the genome" by one of its founders (Lane, 1992).

1.3.1 p53 structure

p53 is a homo-tetramer and contains 393 amino acids. The structure of p53 has been shown in **Figure 1-5**. In order to perform its functions, p53 requires multiple domains which can be divided into three groups: N-terminal domain, Core domain and C-terminal domain.

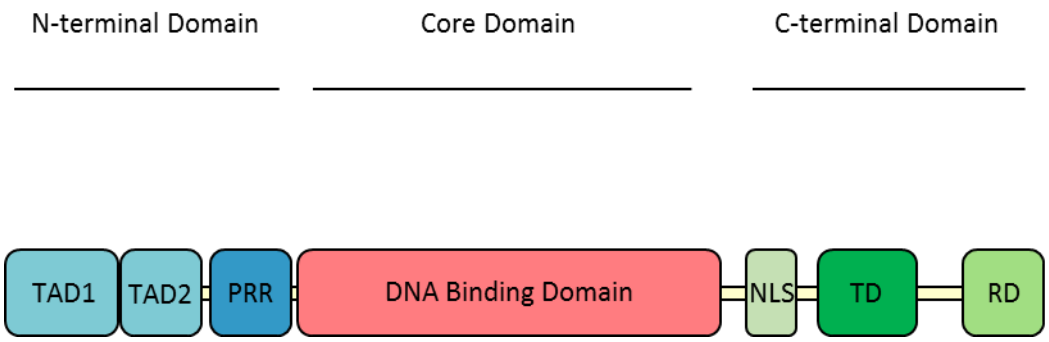


Figure 1-5. Structure of p53 protein.
Schematic represents important domains of p53 protein. TAD1 and TAD2 are transcriptional activation domains, PRR is the proline rich region, NLS is nuclear localisation sequence, TD is tetramerisation domain and RD is the regulatory domain.

The **N-terminal domain** consists of 2 transactivation domains (TAD1 and TAD2) and a proline rich region (PRR). The transactivation domains are required for p53 to function as a transcription factor (Fields and Jang, 1990). The proline rich region has been reported to be required for growth suppression, apoptosis and response to ionising radiation (Campbell *et al.*, 2013).

The **core domain** is responsible for site/sequence specific DNA binding of p53 to its target genes at sequence specific elements, termed p53 responsive elements (p53REs). The majority of cancer-related mutations in the *TP53* gene (encoding p53) occur within this region and are generally missense mutations (Vousden and Lu, 2002). Six hotspot mutations on this domain have been identified. Three of these mutations are 'conformational' mutants which affect the residues that stabilise the tertiary structure of DNA binding surface (Arg 175, Gly245 and Arg 249). The other three are 'contact' mutations that affect the residues necessary for making direct contact with DNA (Arg 248, Arg 273 and Arg 282) (Olivier, Hollstein and Hainaut, 2010; Freed-Pastor and Prives, 2012). Mutations at these sites not only cause loss of wild type p53 functions, but also can cause gain of novel oncogenic functions (Muller and Vousden, 2013).

The **C-terminal domain** comprises sequences for smaller domains and elements. These are nuclear localisation signal, tetramrisation domain and C-terminal regulatory domain. Nuclear localisation signal as the name indicates is required for the localisation of p53 into nucleus. Tetramerisation domain mediates formation of homo and hetero tetramers which are crucial for p53 to perform DNA binding, protein-protein interactions, post translational modification (PTM) and p53 degradation. The C-terminal domain has also been

reported to have a nuclear export signal that regulates p53 degradation (Chène, 2001). The regulatory domain is an important region for a variety of post translational modifications and p53 non-specific binding (Jayaraman and Prives, 1999).

1.3.2 p53 regulation

In unstressed conditions, the cellular levels of p53 are low and the protein undergoes rapid turnover. However, in the event of cellular stresses (DNA damage, hypoxia, telomere erosion, mitotic catastrophe, oncogene activation, etc.) p53 levels increase. This rapid increase could result in different cellular responses such as cell cycle arrest and/or apoptosis.

The main negative regulator of p53 is the ring finger E3 ubiquitin ligase MDM2 (mouse double minute 2). A MDM2 docking site in the transactivation domain of p53 enables specific interaction of p53 and MDM2. This allows MDM2 to ubiquitinate p53 and transport it to cytoplasm for degradation. MDM2 mediated regulation of p53 is part of an auto regulatory negative feedback loop mechanism. The promoter region of the *MDM2* gene contains a p53RE which enables active p53 to induce the expression of its own inhibitor (Honda and Yasuda, 2000). Negative regulation of p53 activity by MDM2 has been shown in mouse models. MDM2 knockout mice are embryonically lethal due to excess apoptosis. The lethality is rescued when p53 is knocked out too (Luna, Wagner and Lozano, 1995).

p53 is tightly regulated by MDM2 and uncoupling/preventing the MDM2-p53 interaction is universally required for p53 stabilisation and activity. This can be

done in several ways. ARF (alternative reading frame) is a protein that, upon its expression, binds to MDM2 central acidic domain and inhibits its ubiquitin ligase function leading to p53 stabilisation (Luna, Wagner and Lozano, 1995). ARF can also sequester MDM2 within the nucleolus, in a physically separate compartment, thereby activating p53 in nucleoplasm (Weber *et al.*, 1999).

DNA damage-induced phosphorylation of MDM2 is another example by which MDM2 mediated ubiquitination of p53 is disturbed. In response to DNA damage (IR), ATM phosphorylates MDM2 at serine 395 which inhibits nuclear export of p53 by MDM2. This leads to accumulation of p53 (Maya *et al.*, 2001).

Apart from ubiquitination, p53 undergoes a wide range of other PTMs which play important roles in p53 regulation. One of the key events disturbing the MDM2-p53 interactions, is phosphorylation of p53. In the event of cellular stress, p53 is phosphorylated at its transactivation domain (including serine 15, Threonine 18 and serine 20). This causes conformational changes in p53 and inhibition of MDM2 binding. p53 phosphorylation at serine 15 has been reported to cause conformational changes in p53, reducing its interaction with MDM2 and increased levels of p53 (Shieh *et al.*, 1997). Role of serine 20 phosphorylation in stability of p53 has been documented too. Mutation of serine 20 to an alanine in p53 led to high sensitivity of p53 to degradation by MDM2 in response to IR and UV light (Chehab *et al.*, 1999). However, another group have reported that phosphorylation of p53 at threonine 18, but not serine 15 and/or serine 20, significantly weakens binding affinity of MDM2 to p53 (Schon *et al.*, 2002). It has also been documented that phosphorylation of residues in p53 N-terminus (serine 15, 33, 37), as the result of cellular stresses, can stabilise p53 binding to p300 and CREB-binding protein (p300/CBP) (Dumaz

and Meek, 1999; Lambert *et al.*, 1998). The transcriptional factor acetyl transferase activity of p300/CBP can acetylate p53 at residues required for MDM2 mediated ubiquitination of p53 which results in abrogation of ubiquitination by MDM2 and stabilisation of p53 (Li *et al.*, 2002).

There are many mechanisms/proteins involved in p53 regulation and its stabilisation in response to different types of cellular stresses which varies depending on the nature of stimuli and cell type. DNA damage is one of the best and most studied triggers of p53 pathway.

1.3.3 p53 pathway and DNA damage

ATM and ATR have an important role in stabilisation of p53 in response to DNA damage. As discussed in the previous section, DNA damage induced-phosphorylation of MDM2 leads to uncoupling of MDM2-p53 and p53 activation. ATM and ATR can phosphorylate p53 at serine 15, a key event in transcription of p53 regulated genes such as p21 (Loughery *et al.*, 2014). Not only ATM and ATR but also DNA-PK (DNA dependent protein kinase) has been suggested to phosphorylate p53 at serine 15 and 37 in response to DNA damage (Lees-Miller *et al.*, 1992).

DNA damage also results in phosphorylation of p53 at serine 20 by Chk1 and Chk2 (downstream targets of ATR and ATM respectively) (Shieh *et al.*, 2000).

Another activator of p53 is p38, belonging to the MAPK (mitogen activated protein kinase) family. In response to UV radiation, p38 phosphorylates p53 at serine 33 and 46 leading to p53 activation (Bulavin, 1999).

p53 activation is responsible for regulating many genes ultimately leading to cell cycle arrest, senescence or apoptosis depending on the level of the damage.

The cell cycle arrest itself could either be permanent or temporary until the damage is repaired.

1.3.4 p53 as a transcription factor

As a transcription factor p53 can mediate transcription of different genes critical for control of the cell cycle and induction of apoptosis (Appella and Anderson, 2001). p53 has been suggested to cause both transactivation and repression of its target genes. The transactivation role of p53 is better known and studied. The main mechanism by which p53 transactivates genes is by direct binding to sequence specific DNA sites called **p53 responsive elements (p53REs)** (Sullivan *et al.*, 2017). p53-mediated gene repression has been suggested to occur by both direct and indirect mechanisms (Riley *et al.*, 2008). However, more recent analysis has suggested that p53 is solely an activator and the repression of genes by p53 could happen only through indirect mechanisms (Fischer, Steiner and Engeland, 2014).

PLK1 is one of the genes that has been reported to be a target of p53 transcriptional repression. Before explaining different mechanisms reported for *PLK1* repression by different laboratories (which will be discussed in section 3.1), a brief introduction is given below about different elements/proteins that are involved in p53-mediated transcriptional activation and repression.

TATA box binding protein (TBP): TATA box is a sequence in the promoter of some genes and indicates (to other proteins) where transcription should initiate. It is generally about 30 base pair (bp) upstream of transcription start site (TSS) and is present in only 10 to 20% of human genes (Kornberg, 2007). It is a

binding site for TATA box binding protein (TBP) and consequently the transcription factor TFIID complex (consists of TBP and several subunits called TATA binding protein associated factors (TAFs) such as TAF1 and TAFII250) (Hernandez, 1993, Burley and Roeder, 1996). TFIID complex will make part of the RNA polymerase II pre-initiation complex and helps in positioning RNA polymerase II over the TSS (Hernandez, 1993). It has been documented that p53 binds to TBP forming p53-TBP complex. The complex can then directly bind to TATA box and promote binding of RNA polymerase and initiate transcription (Seto *et al.*, 1992; Martin *et al.*, 1993; Chen *et al.*, 1993)

CCAAT box binding factor (CBP/NF-Y): The CCAAT box is one of the most prevalent elements in the promoter of eukaryotic cells and its mutation has been documented to cause several fold decrease in transcriptional activity (Maity and De Crombrugghe, 1998). It has been suggested that in TATA-less promoters, there is increased occurrence of CCAAT boxes (Mantovani, 1998). The main protein recognising and binding to this box is NF-Y (nuclear transcription factor Y) (Mantovani, 1999). NF-Y is composed of three subunits; NF-YA, NF-YB, NF-YC. (Maity and De Crombrugghe, 1998). It interacts with different transcription factors and also to histone acetyl transferases (Mantovani, 1999). NF-Y is essential for the recruitment of RNA polymerase II to several CCAAT box containing promoters (Kabe *et al.*, 2005).

NF-Y and CCAAT boxes are important not only for transactivation of genes but also for their repression in the event of DNA damage. It has been reported that p53 can repress promoters of key regulators of the G2/M transition (such as cdc2, cyclin B and CDC25C) by its interaction with NF-Y and CCAAT boxes. p53 rapidly undergoes acetylation following DNA damage. This leads to

recruitment of histone deacetylases on G2/M promoters, deacetylation of histones and release of P300 and PCAF which coincide with repression of the promoters. This can then result in checkpoint arrest (Imbriano *et al.*, 2005).

Indirect p53 repression of genes through this box has been suggested too. The model proposed by Lin *et al*, indicates that in unstressed cells CDK2 phosphorylates nuclear factor YA (NF-YA) subunit of the CCAAT box which is responsible for sequence specific DNA binding. This association facilitates binding of NF-Y and CCAAT box and enables transactivation of *PLK1* which is necessary for cell cycle progression. In stressed conditions on the other hand, activated p53 induces p21. p21 then displaces CDK2 in interacting with NF-YA so there will be less association of CDK2 and NF-YA. This leads to CCAAT box-associated regulatory complex to have repressive function presumably with recruitment of co-repressors or HDACs (Lin *et al.*, 2014).

Specificity protein 1 (SP1): SP1 is a transcription factor that binds to the GC box (GC rich motif) of many promoters. The activity of SP1 as a transactivator or repressor, is affected by post translational modifications. p53 has been shown to have a strong cooperation with SP1 in regulation of several tumour suppressor and oncogenes. This cooperative regulation of transcription by p53 and SP1 varies between genes. Some studies show that they work together to promote transcription while in other instances they demonstrate opposing effects on transcription (Beishline and Azizkhan-Clifford, 2015).

E2F binding site: E2F is a family of transcription factors which bind to the promoter sequences called E2F binding sites. They have been recognised as regulators of transcription of the cell cycle-dependent genes with maximal

expression in S phase. Depending on which member of E2F family bind to the consensus element, the outcome could be activation or repression of the gene (E2F1-3 are activators while F2F4-5 are repressors) (Slansky and Farnham, 1996; Helin, 1998).

p53 can repress genes indirectly through E2F binding sites. It involves p21 upregulation and subsequent CDK1 inhibition which leads to hypo-phosphorylation of p130/107 and formation of inhibitory DREAM complex (Müller *et al.*, 2012; Litovchick *et al.*, 2007). Müller *et al.* showed that in genes mainly expressed in S phase, DREAM binds to E2F sites and the binding can be supported by interaction of LIN54 of MuvB core complex with elements called CHR like elements (CLE) positioned 4 bp downstream of E2F binding site which differ in one or 2 nucleotides from the consensus (Müller *et al.*, 2016).

CDE/CHR elements: As mentioned in section 1.2.5, The cell cycle-dependent element (CDE) and the cell cycle genes homology region (CHR) are elements found in the promoter of cell cycle dependent genes with maximal expression in G2/M. CDE/CHR are responsible for negative regulation of these genes early in cell cycle and mutation of either of these elements results in activation of transcription and loss of cell cycle regulation of these genes (Zwicker *et al.*, 1995; Badie *et al.*, 2000). However, the CDE function has been suggested to be secondary to CHR function (Müller *et al.*, 2012). CDE sites are in fact special E2F binding elements and share similar sequences with them but what distinguishes CDEs is that CDEs are only 4 nucleotides upstream of CHR elements. CDE/CHR containing promoters usually lack a TATA box and contain multiple CCAAT boxes (Müller and Engeland, 2010).

p53 mediates downregulation of genes expressed in G2/M in response to stresses and one of the mechanisms suggested is through CDE/CHR. (Müller and Engeland, 2010). The mechanism is similar to the suggested mechanism for E2F binding sites which involves upregulation of p21 by p53 and formation of DREAM complex which binds to CDE/CHR elements (Müller *et al.*, 2012; Litovchick *et al.*, 2007) . These events could lead to gene repression. p53 has also been reported to mediate downregulation of CDC25C through CDE/CHR elements but independently of p21 as p53 could still repress the promoter in the HCT116 p21 null cells (Clair *et al.*, 2004).

Despite nearly 40 years of research and an ever-growing number of publications on p53 (around 90000 listed in PubMed), its central role as a transcriptional regulator has not yet been understood completely. How p53 acts as a transcriptional activator for one target gene and repressor for another, remains to be resolved. There has been enormous amount of discoveries in p53 field, but the picture is not complete yet. Additionally, some scientists have suggested that p53 could act as both transcriptional activator and repressor and some others believe p53 is solely an activator.

As explained before, several elements/factors have been identified which are involved in p53 regulated transactivation and/or repression. Importantly, there has been some apparently contradictory data reported by different groups, for some genes. For example, in case of *PLK1* repression by p53, several mechanisms have been reported by different groups which seems conflicting. This made the basis of our investigations in chapter 3 of this thesis. The

mechanisms suggested for *PLK1* repression by p53 will be discussed later in the background of the chapter 3 (section 3.1).

1.4 Thesis Aims

Given the importance of PLK1 in proliferation and its many functions especially during mitosis, importantly the high dependency of cancer cells to PLK1, there are two critical areas where further analysis would provide better understanding of how this protein functions and regulated. These are stated in the following aims:

- To investigate whether the recently proposed universal model of p53 mediated repression through regulating DREAM complex explains p53-mediated *PLK1* repression in a satisfactory manner or whether other mechanisms may additionally operate, either through cooperating with DREAM or independently of DREAM perhaps in a context-dependent manner.

- Knowing the important functions of PLK1 in mitosis, attempt to bring more insight into the consequences of pharmacologically inhibiting PLK1 in mitosis. Also, to investigate if there are differences between the consequences of mitotic inhibition by PLK1 inhibition and that induced by microtubule poisons.

Chapter 2: Materials and Methods

2.1 Chemicals

Chemical Name	Manufacturer	Catalogue No.	Use
BI2536	Selleck chemicals	S1109	PLK1 inhibitor
Cisplatin	Sigma Aldrich	479306	interferes with DNA replication
Doxorubicin	Sigma Aldrich	25316-40-9	interacts with DNA by intercalation and inhibits the biosynthesis of macromolecules
Etoposide	Selleckchem	S1225	Topo-isomerase II Inhibitor
Geneticin (G418)	Life Technologies	108321-42-2	Antibiotic (selection for stable lines)
Hygromycin B	Life Technologies	31282-04-9	Antibiotic (Selection of stable lines)
KU55933	Sigma Aldrich	SML1109	ATM inhibitor
Nocodazole	Calbiochem	487928	Prevents microtubule polymerisation
NU6027	Sigma Aldrich	N4411	ATR inhibitor
NU7441	Tocris	3712	DNA-PKc inhibitor
Nutlin-3a	Tocris	3984	Inhibits the interaction between MDM2 and p53

ZM 447439	Selleckchem	S1103	AuroraB inhibitor
zVAD-fmk	Calbiochem	627610	Pan-Caspase inhibitor
5-Bromo-2'-deoxyuridine (BrdU)	Sigma Aldrich	B9285	Thymidine analogue

Table 2.1 List of chemicals used.

2.2 Antibodies

Antibody	Species	Manufacturer and Catalogue No.	Epitope	Dilution
Actin	Rabbit	Sigma Aldrich A2066	Actin C-terminus (C11 peptide) (Polyclonal)	1:2500 (WB)
BrdU	Mouse	Becton Dickinson 347580	Ioduridine (Monoclonal)	1:50 (Flow Cytometry)
Cyclin A	Mouse	BD transduction laboratories 611269	Human Cyclin A aa 26-144 (Monoclonal)	1:1000 (WB)
Firefly Luciferase	Rabbit	Abcam Ab99944	Anti-Firefly Luciferase (Polyclonal)	1:1000 (WB)

GAPDH	Mouse	Sigma Aldrich G8795	Anti-GAPDH antibody (Monoclonal)	1:5000 (WB)
ICAD	Mouse	Santa Cruz biotechnology sc-17818	ICAD antibody against aa 1-331 (representing full length ICAD of human origin) (Monoclonal)	1:1000 (WB)
MDM2	Mouse	Moravian 4B2	Human MDM2 N-terminus (Monoclonal)	1:500 (WB)
p21 (H-164)	Rabbit	Santa Cruz Biotechnology sc-756	Human p21 aa 1-164 (Polyclonal)	1:500 (WB)
p53 (1801)	Mouse	Santa Cruz Sc-98	Human p53 N-terminus (aa 46-55) (Monoclonal)	1:800 (WB)
p53 (DO1)	Mouse	Santa Cruz Biotechnology sc-126	Human p53 N-terminus, aa 20-25 (Monoclonal)	1:1000 (WB)
p53 (Sapu)	Sheep	Kindly provided by Dr. JC Bourdon (Khoury and Bourdon, 2010)	Human p53 recognising epitopes at the p53 N-terminus (1-92), DBD (280-290) and C-terminus (340-393) (polyclonal)	1:5000 (WB)
p53 pS15	Rabbit	Cell Signaling Technology	Human p53 with phosphorylated serine 15 residue	1:1000 (WB)

		9284	(Polyclonal)	
phospho histone H3 (ser10)	Mouse	Millipore 06570	Histone H3 with phosphorylated Serine 10 residue (Polyclonal)	1:1000 (IF)
PLK1	Rabbit	Cell Signaling Technology 4513	Human PLK1 C-terminus (Monoclonal)	1:1000 (WB)
TRF2	Mouse	Millipore 05-521	TRF2 Antibody, clone 4A794 (Monoclonal)	1:1000 (IF)
γ-H2AX (pS139)	Rabbit	Santa Cruz sc-101696	Human γ-H2AX with phosphorylated serine 139 residue (Polyclonal)	1:1000 (IF)
γ-H2AX (pS139)	Mouse	Millipore 05-636	Human γ-H2AX with phosphorylated serine 139 residue (Monoclonal)	1:1000 (IF)
γ-H2AX (pS139)	Rabbit	Abcam Ab11174	Human γ-H2AX with phosphorylated serine 139 residue (Polyclonal)	1:5000 (WB)
53BP1	Rabbit	Novus biological NB100-304SS	human 53BP1 aa 350-400 (Polyclonal)	1:1000 (IF)

Table 2.2 List of Primary antibodies used.

Secondary Antibody	Species	Manufacturer	Catalogue Number	Dilution
HRP-linked anti-mouse	Goat	Biorad	172-1011	1:2000 (WB)
HRP-linked anti-rabbit	Goat	Biorad	170-6515	1:2000 (WB)
HRP-linked anti-Sheep	Rabbit	Jackson immunoresearch	170-6515	1:5000 (WB)
Alexa-488–conjugated anti-mouse	Goat	Invitrogen	A-11001	1:500 (IF)
Alexa-488–conjugated anti-rabbit	Goat	Invitrogen	A-11034	1:500 (IF)
Alexa-594–conjugated anti-mouse	Goat	Invitrogen	A-11005	1:1000 (IF)
Alexa-594–conjugated anti-rabbit	Goat	Invitrogen	A-11012	1:1000 (IF)

Table 2.3 List of secondary antibodies used.

2.3 Reagents and Buffers

Unless otherwise stated all chemicals were purchased from Sigma Aldrich.

Freezing media:

90% FBS

10% DMSO

Immuno-Fluorescence (IF) Reagents and Buffers:

Antibody dilution buffer

20 mM Tris pH7.5

150 mM NaCl

2% (w/v) BSA

2% (w/v) marvel

0.2% (w/v) fish gelatine

0.1% (v/v) triton X100

KCM buffer:

120 mM KCL

20 mM NaCl

10 mM Tris pH 7.5

0.1% (v/v) triton X100

Hypotonic buffer:

20 mM Hepes pH 7

1 mM MgCl2

20 mM KCL

10 µl 1M CaCl2

PNA hybridisation solution:

70% (v/v) deionised formamide

0.25% (v/v) blocking reagent 3GM

10 mM Tris pH7.5

4 mM Sodium orthophosphate

0.5 mM citric acid

12.5 mM MgCl2

PNA wash A:

70% (v/v) deionised formamide

10 mM tris pH 7.5

PNA wash B:

50 mM tris pH 7.5

150 mM NaCl

0.08% Tween 20

Western Blotting Reagents and Buffers

Enhanced chemiluminescence (ECL) solution 1

2.5 mM Luminol

100 mM Tris (pH 8.5)

396 µM P-Coumaric Acid

Enhanced chemiluminescence (ECL) solution 2

0.0192% (v/v) H_2O_2

100 mM Tris (pH 8.5)

Ponceau S staining buffer

0.2% (w/v) Ponceau S

5% glacial acetic acid

SDS protein sample buffer (2X)

0.125M Tris-HCl (pH 6.8)

20% (v/v) Glycerol

4% (w/v) SDS

0.02% (w/v) Bromophenol Blue

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Running Buffer (10X)

250 mM Tris

1.92 M Glycine

1% (w/v) SDS

Western Blot Blocking Buffer and antibody dilution buffer

1X PBS

0.1% (v/v) Tween 20

5% (w/v) Marvel Dried Milk

Western Blot Transfer Buffer

25 mM Tris

192 mM Glycine

20% (v/v) Methanol

Western blot washing buffer

1X PBS

0.1% (v/v) Tween 20

Flow Cytometry Reagents and Buffers

Propidium iodide staining buffer

50 µg/ml Propidium Iodide

200 µg/ml RNase A

1X PBS

Antibody dilution buffer for flow cytometry

1x PBS

0.5% (w/v) Bovine Serum Albumin (BSA)

0.5% (v/v) Tween 20

2.4 Cell Culture and Related Methodologies

2.4.1 Cell culture maintenance

Stocks of all cell lines were available in the laboratory (see table 2.4 for sources) and were grown in Dulbecco's Modified Eagles Medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS, Biosera (Labtech International Ltd.)) and 2 mM L-glutamin (Gibco) and were maintained at 37°C in a humidified atmosphere set at 5% CO_2. When cells were about 70-90% confluent, media was aspirated, adherent cells were washed twice with PBS and then trypsinised (using 0.05% trypsin-EDTA dissociation reagent) to detach

the cells from the flask. Detached cells were then re-suspended in media and sub-cultured.

Cell Line	Derivation	ATCC® Number
U2OS	Human osteo-sarcoma-ARF null	HTB-96™
A549	Human Non-small cell lung carcinoma	CCL-185™
H1299	Human non-small cell lung carcinoma-p53 null	CRL-5803™
HCT116 (wild type)	Human colorectal carcinoma	CCL-247™
HCT 116 (p53 null)	Human colorectal carcinoma	Vogelstein Laboratory*
HCT116 (p53 null, p21 null)	Human colorectal carcinoma	Vogelstein Laboratory*

*Dr. Vogelstein, Johns Hopkins University, USA

Table 2.4 Mammalian cell lines used

2.4.2 Cryopreservation of cells

Cells were seeded in a 175 cm^2 flasks and when they reached about 80% confluency, they were trypsinised and re-suspended in media. The suspension was then centrifuged at 300 xg for 5 minutes to obtain the pellet. After centrifugation, growth media was aspirated, and pellet was re-suspended in 5 ml of ice-cold freezing media. Cell suspension (1ml) was then transferred to

each cryogenic vials (corning). Vials containing cells were then transferred to an insulated polystyrene box to ensure slow freezing and were then transferred to -80°C freezers for a few days before transferring them to liquid nitrogen for long term storage.

2.4.3 Revival of cryopreserved cells

Cryogenic vials were taken from liquid nitrogen and placed in dry ice. Vials were then placed in a water bath set at 37°C for a quick thaw. Cell suspension was then transferred to pre-warm growth media (37°C). Cells were then centrifuged at 300 xg, media was aspirated, and pellet were re-suspended in fresh media and transferred to a flask and incubated in 37°C, 5% CO2 in humidified atmosphere.

2.4.4 Cell counting and seeding

Trypsinised cells were pooled together and re-suspended in media. 10 µl of this suspension were loaded to each side of a haemocytometer (Hawksley, Sussex, UK, 1/400 m^2). Cells were then counted under microscope using a M45 Hand Tally (ENM counting instrument, Chicago, USA) and seeded as required.

2.4.5 Drug treatments

Cells were seeded at the appropriate density according to the size of the plate or dish and incubated overnight in 37°C, 5% CO_2, in humidified atmosphere. Following overnight incubation different concentrations of drugs were added to

the cells and incubated for further 24 hours. As control, cells were either untreated or treated with 0.1% dimethyl sulfoxide (DMSO, used as the vehicle for drugs), in case DMSO alone has any effect on the cells.

2.4.6 Transient DNA transfections

Cell lines to be transfected were seeded in appropriate density (around 60% confluent at the time of transfection) and incubated overnight before transfection. Transfection with plasmid DNA was then carried out using either FuGENE HD (Promega) or Lipofectamin 2000 (Invitrogen). Where different amounts of a gene were added, the volume topped up with empty vector so that there is equal amount of the plasmid transfected in all conditions of an experiment. Transfected cells were then incubated for another 24 hours before 1X passive lysis buffer (Dual Luciferase Reporter Assay System, Promega) was used to lyse the cells. Each transfection was carried out in triplicate.

2.4.7 Gene silencing using siRNA

Cells were transfected with siRNA by reverse transfection using Lipofectamine® RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's protocol. The siRNAs used are given in **table 2.5**. In each 6 well dish 1.5 µl of 20 µM siRNA oligo was added to 500 µl Opti-MEM and mixed gently. 5 µl RNAiMAX was then added to each well and incubated for 20 minutes at room temperature. $2.5x10^5$ cells suspended in 2.5 ml of media was added to each well following 20 minutes incubation and mixed to distribute evenly. Cells were

then incubated for 24 hours before further experimentation carried out. Non-silencing siRNA was used as a control.

siRNA	Target sequence	Manufacturer	Catalogue No.
$p21^{WAF1}$	5'-CGACUGUGAUGCGCUAAUG-3' 5'-CCUAAUCCGCCCACAGGAA-3' 5'-CGUCAGAACCCAUGCGGCA-3' 5'-AGACCAGCAUGACAGAUUU-3'	Dharmacon	L-003471-00-0005
p53 (exon7)	5'-GACUCCAGUGGUAAUCUACUU-3'	Thermo Scientific	OSLR-001137
Non-silencing (NS)	5'-CAGUCGCGUUUGCGACUGG-3'	Thermo Scientific	OSLR-001139

Table 2.5 List of siRNAs used.

2.4.8 Luciferase reporter assay

Cells were seeded in 12 or 24 well plate at appropriate density and transfected using FuGENE HD or Lipofectamine 2000. Cells were transfected with a pGL3 plasmid in which 2.4 Kb of the human *PLK1* promoter was cloned upstream of the *firefly luciferase* gene. Transfected cells were incubated for 24 hours before any further experimentation carried out. Cells were harvested 24 hours after treatments using passive lysis buffer (Promega). Dual luciferase reporter assay system (Promega) was used to analyse 20 µl of protein extracts in each well of

a 96 well plate by luminometer (GloMax®-Multi Detection System (Promega)). Transfection efficiencies were normalised by using Renilla luciferase activity of the extracts or by doing protein quantification using DC (detergent compatible) assay followed by western blot analysis to check the efficiency of the transfection.

2.4.9 Construction of stable lines

U2OS cells were seeded at the density of $4x10^4$ cells/well in a 6 well plate. Following overnight incubation cells were treated with either pGL3 plasmid (basic, Promega) or pGL3 plasmid in which 2.4 kb of the human wild type *PLK1* promoter or *PLK1* promoter that is mutated at CDE/CHR elements cloned upstream of the *firefly luciferase* gene **(figure 2.1)**. pcDNA3 plasmid was co-transfected at the ratio of 1:10 (pcDNA3:pGL3) as a selection marker as it (pcDNA3) has a G418 resistant gene. Cells were kept in media containing G418 (400 µg/ml) until surviving cells formed colonies. Colonies were then isolated and re-plated in media containing G418. After cells reached enough confluency, samples were harvested and tested with luciferase assay to screen for positive colonies.

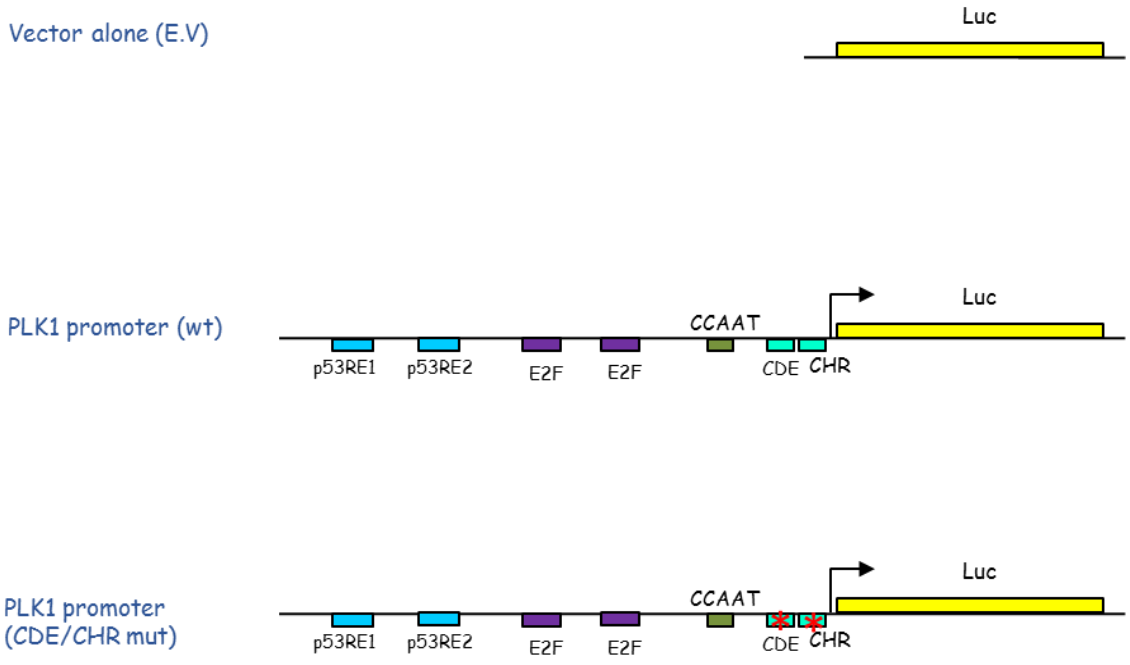


Figure 2-1. Schematic representation of the different constructs cloned in pGL3 plasmids and transfected into the cells.

2.4.10 Mitotic wash off and preparation of metaphase spreads

Cells were seeded and incubated overnight at 37ºC. Cells were then synchronised by nocodazole/PLK1 inhibitor for 2 hours. Mitotic shake off was carried out to collect the mitotic cells. This was done by applying slight mechanical force, by pipetting up and down the media, to separate the loosely attached rounded up cells. Rounded up cells/floating cells in the growth media were then collected, leaving adherent cells behind. Following this, pre-synchronised cells were either pelleted and processed for further experimentation or re-plated in nocodazole/PLK1 inhibitor for a prolonged mitotic arrest (further 2 or 4 hours). Normal mitotic cells were collected from untreated asynchronous cells. Cell suspension was then centrifuged at 300 xg for 4 minutes at room temperature. Mitotic cell suspension was then washed and centrifuged again, media was removed leaving around 50 µl behind. Cells were then suspended, in the remaining 50 µl of the media, and hypotonic buffer was added and mixed with gentle shaking (without the use of pipettes). Cell suspension in hypotonic buffer was incubated at room temperature for 10 minutes.

Using cytospin apparatus cells were then centrifuged onto super frost plus glass slides and centrifuged at 300 xg for 3 min after initial equilibrisation of the system which included loading 100 µl of 2% BSA in the cytospin and centrifuging for 1 min at 300 xg. Slides containing metaphase spreads were left on bench for about one minute to air dry and then were fixed using 4% formaldehyde in PBS for 10 minutes at room temperature. Slides were then rinsed few times with PBS and proceeded with next steps or stored in 4°C.

2.4.11 Clonogenic assay

A549 or U2OS cells were seeded in appropriate density in 10 cm dishes and incubated overnight at 37ºC. Following overnight incubation cells were treated with nocodazole or PLK1 inhibitor. Mitotic shake off was carried out. Mitotically arrested cells were then counted and re-plated in fresh media at the density of $1.5x10^3$ cells per 10 cm dish. Dishes containing cells were then incubated for 9 days when colonies reached desired size. Clonogenic assays were carried out by washing the dishes containing colonies twice with PBS. They were then fixed by exposing them with methanol for 10 min. Methanol was then aspirated from the plate and replaced with 0.4% crystal violet solution (made in 25% methanol) for staining. After 10 minutes, the crystal violet solution was aspirated. Plates were then immersed in running water and rinsed carefully until colour no longer come off in the rinse. Plates were then left to air dry and Number of survived colonies in each dish was then counted.

2.5 Preparation and Quantification of Protein Samples

Cells were washed twice with PBS and lysed by using 2X SDS page sample buffer using cell scrapers. Lysates were then sonicated for homogenisation for 20 seconds at 30% amplitude. Total protein in each sample was quantified by DC assay kit (Biorad), according to manufacturer's protocol. Briefly reagent A and S were mixed at the ratio of 50:1. 25 µl of the mix was then transferred to each well of a 96 well plate along with 17 µl of distilled water. 3 µl of lysates were then added to the wells and then 200 µl of reagent B added to all the wells containing lysates. Everything was conducted in duplicate. The plate was then

incubated for 15 minutes at room temperature in dark before absorbance was read at 750 nm using GloMax®-Multi Detection System (Promega).

2.6 Separation of Proteins Using SDS-PAGE

Prepared samples were heated at 90ºC for 5 minutes. Unless otherwise stated in the figure legends, 20 µg of proteins were loaded in appropriate percentage of acrylamide gels (table 2.6 and 2.7). PageRuler™ Plus Prestained Protein Ladder 10-250 KDa (Thermo Fisher Scientific) was loaded in gels adjacent to the samples to determine the molecular weight. Proteins were separated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), using a vertical Slab Electrophoresis Chamber Apparatus (Atto) and SDS-PAGE running buffer. Electrophoresis was carried out using a power supply set at 100 V for the duration of the gel running.

Reagents	8%	10%	12.5%
Distilled water (ml)	3.95	3.3	2.475
12.5 mM Tris-HCL pH 8.8	3	3	3
30% (w/v) Acrylamide 0.8% (w/v) Bis-acrylamide stock solution (37:5:1) (ml)	2.64	3.3	4.125
10% SDS (µl)	100	100	100
10% APS (µl)	100	100	100
TEMED (µl)	10	10	10

Table 2.6 Buffers and their quantity for resolving gels.

Reagents	Volume Required
Distilled water (ml)	2.15
12.5 mM Tris-HCL pH 6.8	0.3
30% (w/v) Acrylamide 0.8% (w/v) Bis-acrylamide stock solution (37:5:1) (ml)	0.5
10% SDS (µl)	30
10% APS (µl)	25
TEMED (µl)	2.5

Table 2.7 Buffers and their quantity for stacking gels.

2.7 Western Blot Analysis

After separation of proteins by SDS-PAGE, proteins were transferred to a Hybound ECL nitrocellulose membrane (Amersham) using wet transfer apparatus (Bio-Rad) at 100 V for 1 hour. Membranes were then blocked in western blocking buffer for one hour at room temperature. They were then incubated in primary antibodies diluted in western blot antibody dilution buffer overnight at 4°C. Following 3 washes with PBS 0.1% Tween-20, appropriate horseradish peroxidase-conjugated secondary antibodies were added to the membranes and incubated for 1 hour at room temperature. Membranes were then washed 3 times, equal volume of ECL1 and ECL2 was then mixed and added to the membranes and proceeded for chemiluminescence detection using ChemiDoc™ MP Imaging System (Bio-Rad).

2.8 Cell Cycle Analysis

Cells were seeded in appropriate density and incubated overnight at 37ºC. Cells were then treated with different agents as indicated in each experiment and incubated for 24 hours. Cells were labelled with 30 µM BrdU and incubated in 37ºC for 30 minutes. BrdU is a thymidine analogue and labels newly synthesized DNA in S phase. A non-BrdU labelled sample was prepared as well as a control. Both floating and adherent cells were collected. Cell pellet was prepared using centrifugation at 300 xg for 5 minutes. Cells were then re-suspended in PBS to wash and pelleted again before re-suspending them in 1 ml of PBS. Cells were then fixed by adding 3 ml of 100% ice cold ethanol dropwise while vortexing. Fixed cells were incubated in 4 ºC for maximum of 2 weeks.

3-4 ml PBS supplemented with 1% FBS was added to the cells and centrifuged for 5 minutes. The pellet was then washed with PBS-FBS and centrifuged. This time cells were treated with 1 ml of 2 M HCl and incubated for 20 minutes at dark in a water bath set at 37ºC. Occasional mixing was carried out during incubation. Wash step with PBS-FBS was then performed twice. Pellets were re-suspended in 200 µl Beckton Dickinson anti BrdU antibody (BD Bioscience, Oxford, UK) diluted 1:50 in antibody buffer and incubated for 1 hour at room temperature in dark with occasional shaking.

Cell suspension was washed with PBS-FBS and pelleted again. Pellet was re-suspended in 200 µl of FITC conjugated anti mouse IgG (whole molecule) antibody diluted to 20 µg/ml in antibody dilution buffer (1:64 dilution) followed by incubation for 30 minutes.

Cell wash step with PBS-FBS was repeated and pellets were re-suspended in 300 µl propidium iodide staining buffer. Cell suspension was then transferred to FACS tubes and incubated at room temperature in the dark for 20 minutes before analysis or stored at 4ºC overnight for analysis by flow cytometer (BD Biosciences). Flowjo software was then used for the analysis of the cell cycle.

2.9 Immuno-Fluorescence Studies and Microscopy

Cells or metaphase spreads were fixed in 4% formaldehyde in PBS for 10 minutes at room temperature and rinsed in PBS. Cells were permeabilised with KCM buffer for 10 minutes and then blocked for 20 minutes at 37°C. Cells were then incubated overnight at 4°C in primary antibodies diluted in blocking buffer. Following three 5 minutes washes with 0.1% Tween-20/PBS secondary antibodies and DAPI were added in blocking buffer and incubated for 1 hour at room temperature in the dark. Then cells were washed 3 times for 5 minutes with 0.1% tween-20/PBS and mounted using mounting media (DAKO). Images of the cells were taken by using a Leica SP5II laser scanning confocal microscope with a HCX Pl Apo CS 63 × 1.4 lens. Minimum of 25 cells were analysed per treatment.

2.10 Fluorescence in Situ Hybridisation (FISH)

Fluorescence in situ hybridisation (FISH) using PNA probe was used for telomere detection after γ-H2AX staining where needed. After 3 washes following secondary antibody, cells were fixed again in 4% formaldehyde in PBS for 10 minutes at room temperature. Fixed cells were then rinsed with PBS and

dehydrated by graded ethanol series, first with 70% ethanol for 3 minutes, then with 90% ethanol for 2 minutes and finally with 100% ethanol for 2 minutes. Slides were then air dried.

Metaphase spreads were stained with PNA probe (Alexa 488–OO-$(TTAGGG)_3$ PNA probe, Panagene) in PNA hybridisation solution which has been preheated at 90ºC (1:100 of 100 nM probe, final concentration of 1 nM). Area of the slides containing metaphase spreads was then treated with PNA probe-PNA hybridisation solution and covered with an 18X18 mm coverslips. Slides were transferred to a pre-heated incubator at 80ºC for 10 minutes. Slides were then incubated at dark in room temperature for 2 hours for hybridisation.

100 ml of PNA wash B was heated in microwave for 2 minutes and poured in coplin jar at 60ºC in the hybridiser with shaking. After 2 hours of incubation slides were washed in PNA wash A to remove the coverslips. Slides were then left in PNA wash A for 5 minutes. They were then washed in pre-heated PNA wash B at 60ºC in the incubator with shaking for 10 minutes followed by another wash with PNA wash B for 2 minutes at room temperature. Metaphase spreads were counter-stained with 250 ng/ml of DAPI in PNA wash B at room temperature for 10 minutes. Slides were then washed with PNA wash B for 5 minutes at room temperature and rinsed with deionised water and mounted in mounting media (DAKO). Slides were progressed for imaging using a Leica SP5II laser scanning confocal microscope with a HCX Pl Apo CS 63 × 1.4 lens. Co-localisation between γ-H2AX foci and telomeres were analysed in minimum of 25 cells in each condition of an experiment.

2.11 Site Directed Mutagenesis

Site directed mutagenesis was carried out using QuickChange Lightning Site Directed Mutagenesis Kit to introduce mutations at a specific sequence of the DNA, according to the manufacturer's protocol. The plasmids in which mutations were introduced were *PLK1* promoter in pGL3 basic plasmid and two truncated versions of it, one having a deletion in p53RE1 and another one deletion of both p53RE1 and p53RE2 (**figure 2.2**). Plasmids were previously prepared by a past member of our laboratory (Iyer *et al.*, 2014).

Newly prepared plasmids (with desired mutations) were sent for sequencing at the Tayside Centre for Genomic Analysis. The sequences were then analysed to check the accuracy of the mutations introduced.

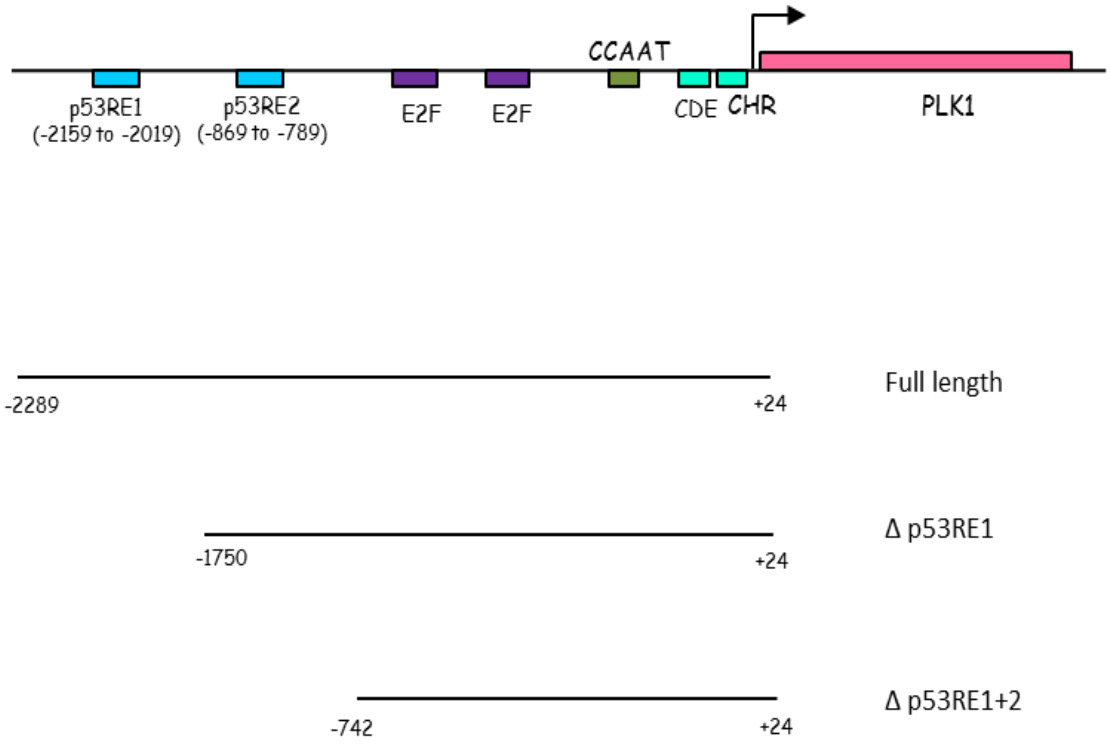


Figure 2-2. Schematic representation of the plasmids used for mutagenesis

For each reaction to be carried out, 100 ng of plasmid DNA was added to 5 µl of 10X reaction buffer along with 125 ng of each forward and reverse primers (table 2.8). 1 µl of dNTP mix and 1.5 µl of QuikSolution reagent were added too and final volume was made up to 50 µl. 1 µl of QuikChange Lightning Enzyme was then added which is a derivative of *PfuUltra* high-fidelity DNA polymerase. PCR was then carried out as shown in Table 2.9.

2 µl of the Dpn 1 restriction enzyme was added after the PCR cycles, mixed thoroughly and incubated at 37ºC for 15 minutes to digest the un-mutated parental (template) plasmid DNA. 2 µl of the digestion reaction was then transformed into XL10-Gold ultracompetent cells.

Primer for Mutagenesis	Sequence
DMCDECHR5	5'-GTAACGTTCCCATATCCGCGTTCACATTCGGGGAGGAG-3'
DMCDECHR3	5'-CTCCTCCCCGAATGTGAACGCGGATATGGGAACGTTAC-3'

Table 2.8 Primers used in mutagenesis.

Segment	Cycle	Temperature (ºC)	Time
1	1	95	2 minutes
2	18	95	20 seconds
		60	10 seconds
		68	30 seconds/kb of plasmid length
3	2	68	5 minutes

Table 2.9 PCR cycles used in mutagenesis.

2.12 Transformation of Competent Cells

45 µl of XL 10-Gold Ultracompetent cells were added to a pre-chilled 14 ml BD falcon polypropylene round bottom tube. 2 µl of XL 10-Gold β-mercaptoethanol (β-ME) mix was added and cells were incubated for 2 minutes on ice. 2 µl of Dpn 1 treated cells was added to the competent cells and mixed gently before incubating it on ice for 30 minutes. Following incubation, the cells were heat-pulsed in a 42ºC water bath for 30 seconds and then incubated on ice for 2 minutes. 500 µl of pre-heated (at 42ºC) S.O.C medium (Invitrogen) was then added to the tubes and incubated at 37ºC for 1 hour with shaking.

After one hour of incubation, 100 µl of the transformation mix was spread evenly over LB-Agar plates containing Ampicillin. Following overnight incubation, individual colonies were picked and added to LB for overnight growth at 37ºC.

2.13 Preparation of Mini-Preps

QIAprep® Spin Mini-prep Kit was used to prepare mini-preps according to manufacturer's protocol.

2.14 Preparation of Maxi-Preps

PureLink® HiPure Plasmid DNA Purification Kit from Invitrogen was used for maxiprep procedure. Following overnight growth of transformed E. coli in LB medium, centrifugation at 4000 xg was performed for 10 minutes. Pellet was then resuspended in 10 ml resuspension buffer containing RNase A. 10 ml of lysis buffer was then added, mixed gently, and incubated for 10 minutes. 10 ml of precipitation buffer was added next and mixed immediately by inverting the tube until a homogenous mixture obtained. Lysate was then centrifuged at 12000 xg for 10 minutes at room temperature. The supernatant was transferred to an equilibrated HiPure Maxi Column and solution was allowed to be drained by gravity flow to allow binding of the DNA to the column. The column was washed with 60 ml of wash buffer and the flow through was discarded. A sterile 50 ml centrifuge tube was placed under the column and 15 ml of elution buffer was added to the column and left to drain by gravity flow so that the purified DNA gets collected on the elution tube. Isopropanol was then added to the elute to precipitate the DNA. The tube was then centrifuged at 12000 xg for 30 minutes at 4ºC. Supernatant was discarded, 70% ethanol was added to the pellet and centrifuged for 20 minutes at 4ºC. Supernatant was discarded, and pellet was allowed to air dry before resuspending purified plasmid in TE buffer. Plasmids were then stored in -20ºC until further use.

2.15 Glycerol Stocks

Glycerol stocks were prepared by adding 800 µl of the overnight culture and 300 µl of 100% glycerol to a cryovial. The cryovial was then stored at -80ºC.

2.16 Statistical Analysis

Where necessary to compare and determine the significance of differences between 2 samples, Student's t-tests were performed. It compares the actual difference between two means in relation to the variation in the data (expressed as the standard deviation of the difference between the means). Results can be considered significant if analysis by Student's t-test resulted in a p-value less than 0.05.

Chapter 3: p53-Dependent Repression of *PLK1* is Mediated through Distinct but Cooperating Pathways

3.1 Background

As mentioned in the introduction chapter (section 1.2.7), several studies have demonstrated downregulation of PLK1 following induction of p53 (McKenzie *et al.*, 2010; Fischer; Quaas, Nickel, *et al.*, 2015; Zhou *et al.*, 2013; Lin *et al.*, 2014). Different mechanisms have been suggested by different groups for this downregulation. These are as follows.

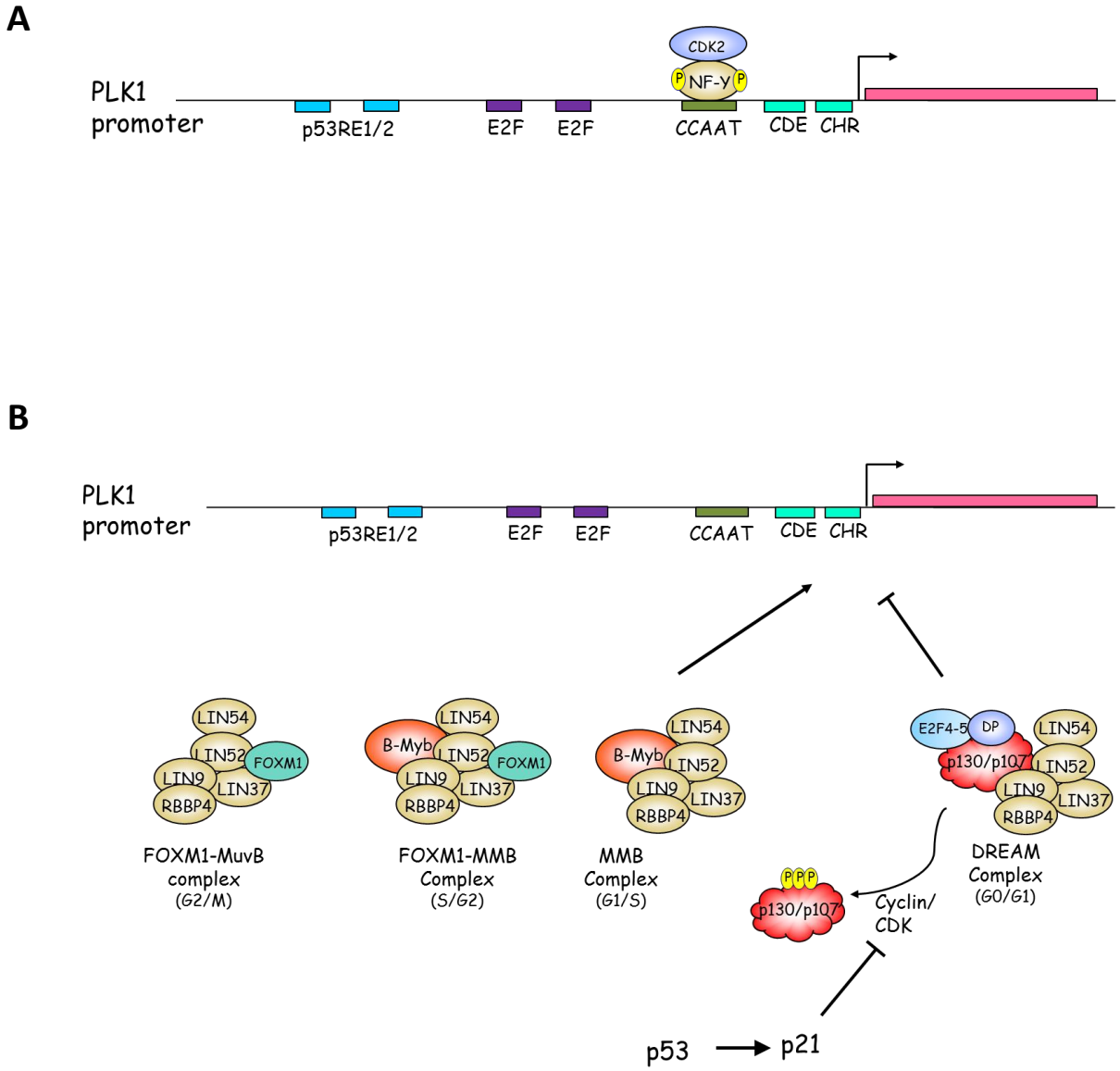
McKenzie et al suggested a direct mechanism; their chromatin immuno-precipitation (ChIP) analysis showed that p53 recruits to p53 responsive elements in the promoter of the *PLK1* in response to DNA damage or p53 activation which causes downregulation of PLK1. They also found that histone deacetylases are involved in this process which further confirms that there is transcriptional repression mechanism (McKenzie *et al.*, 2010) **(Figure 3-1 A)**.

More recently Fischer and colleagues showed that p21 is important in p53 mediated repression of *PLK1*. They showed that CDE/CHR elements of the *PLK1* promoter are needed for this repression. In this model p53 induced p21 blocks the CDK-mediated phosphorylation of RB-related pocket proteins p107/130, keeping them in hypo-phosphorylated state. In this state, p107/130 can join other proteins and form DREAM complex. Association of DREAM with CDE/CHR elements inhibits *PLK1* transcriptional activation (Fischer, Quaas, Nickel, *et al.*, 2015) **(Figure 3-1 B**).

Other elements reported to be involved in *PLK1* repression by p53 such as E2F binding sites. There are two potential E2F binding sites in addition to the CDE/CHR elements in *PLK1* promoter. Zhou *et al.*, 2013 showed that in response to cisplatin induced DNA damage, p53 interacts with E2F1 and forms

an inhibitory p53–E2F1–DNA complex on the *PLK1* promoter and suppress E2F1 mediated *PLK1* expression (Zhou *et al.*, 2013) **(Figure 3-1 C).** On the other hand, another paper suggested that genes with maximal expression in G2/M have been suggested to be devoid of functional E2F binding sites (Müller *et al.*, 2016). Also, Zhu *et al.* showed that E2F binding site deletions do not play a role in *PLK1* promoter regulation (Zhu *et al.*, 2002). Further investigations are needed in this regard to decide whether E2F binding sites are involved.

Many G2/M genes have several CCAAT boxes. p53 can directly repress the promoters of those genes by forming a complex with NF-Y A and NF-Y C. *PLK1*, however, has only one CCAAT box (Imbriano *et al.*, 2005). ChIP analysis could not detect p53 within the core promoter region containing the CAATT box of the *PLK1* promoter (McKenzie *et al.*, 2010). Consistent with that, Lin et al suggested a mechanism for regulation of CCAAT box containing promoters like *PLK1* and *CDC25A*. They suggested that in unstressed cells CDK2 phosphorylates nuclear factor YA (NF-YA) subunit of the CCAAT box which is responsible for sequence specific DNA binding. This association facilitates binding of NF-Y and CCAAT box and enables transactivation of *PLK1* which is necessary for cell cycle progression. In stressed conditions on the other hand, p53 is activated which in turn induces p21. p21 then displaces CDK2 in interacting with NF-YA so there will be less association of CDK2 and NF-YA. This leads to CCAAT box-associated regulatory complex to have repressive function presumably by recruiting co repressors and HDACs (Lin *et al.*, 2014) (**Figure 3-1 D**).


A
PLK1
promoter
p53RE1/2
E2F
E2F
CDK2
NF-Y
CCAAT
CDE
CHR
B
PLK1
promoter
p53RE1/2
E2F
E2F
CCAAT
CDE
CHR
LIN54
LIN52
FOXM1
LIN9
LIN37
RBBP4
FOXM1-MuvB
complex
(G2/M)
B-Myb
FOXM1-MMB
Complex
(S/G2)
MMB
Complex
(G1/S)
E2F4-5
DP
p130/p107
DREAM
Complex
(G0/G1)
Cyclin/
CDK
p53
p21
C
p53
E2F1
PLK1
promoter
p53RE1/2
E2F
E2F
CCAAT
CDE
CHR

D

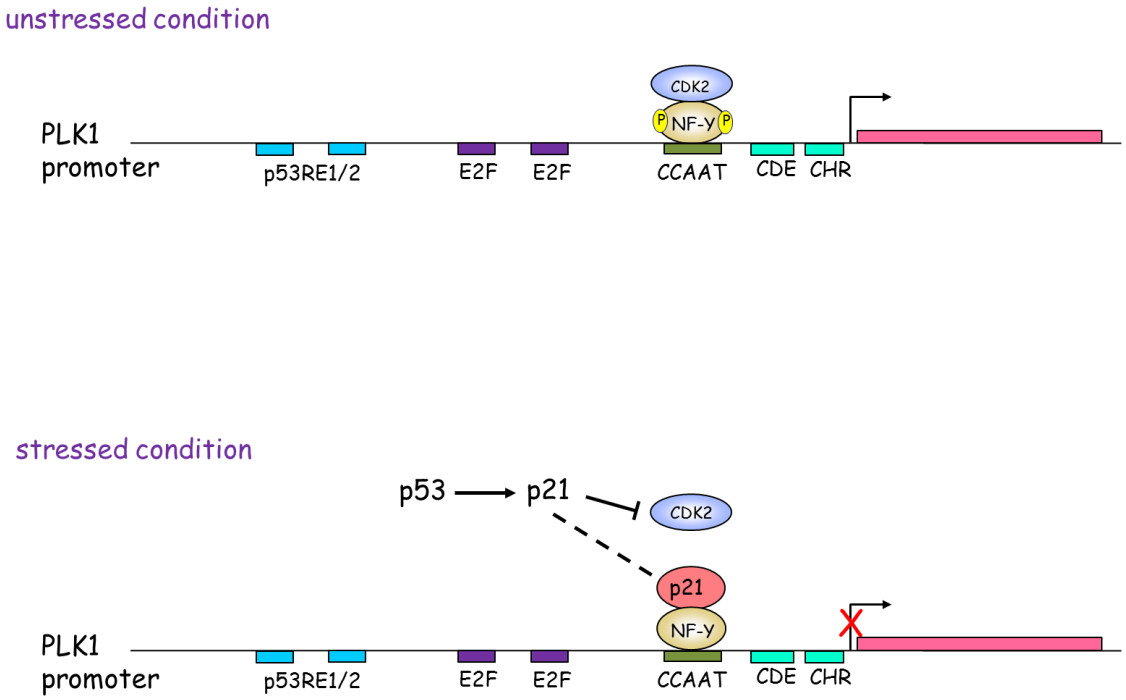


Figure 3-1. Schematic representations of different mechanisms suggested for *PLK1* repression by p53. Different mechanisms of repression of *PLK1* by p53, proposed by different laboratories, are depicted**. (A)** In response to p53 activation, p53 binds to p53REs of *PLK1* promoter and mediates its downregulation directly. **(B)** p53 induces its downstream target, p21, a CDK inhibitor. This blocks the CDK-mediated phosphorylation of RB-related pocket proteins (p107/130), keeping them in hypo-phosphorylated state. As a result, p107/130 can join other proteins and form the inhibitory DREAM complex and subsequently *PLK1* repression occurs. **(C)** p53 interacts with transcription factor E2F1 and forms an inhibitory p53–E2F1–DNA complex on the *PLK1* promoter which results in suppression of E2F1 mediated *PLK1* expression. **(D)** p53 activation induces p21 which in turn inhibits CDK2-NF-Y interaction. NF-Y then binds to p21 and anchors it to CCAAT box, leading to *PLK1* transcriptional repression.

The most recent of these proposed mechanisms (p53-p21-DREAM-CDE/CHR) may universally explain p53 mediated repression of a large set of genes including *PLK1* (Fischer, Quaas, Steiner, et al. 2015). However, given that there are different (and apparently conflicting) mechanisms suggested by different groups and data from our own group showing that under certain circumstances *PLK1* can still be repressed by p53 after elimination of p21, we were interested to further investigate this matter in greater depth.

To resolve this issue, we either used Nutlin-3a or DNA damaging agents to induce p53. **Nutlin-3a** (hereafter Nutlin for simplicity) is a potent and selective small-molecule MDM2 antagonists. It binds to the p53 pocket of MDM2 and mimics the molecular interactions of 3 hydrophobic residues on p53 which are required for binding to MDM2. Thus, Nutlin works as a competitive inhibitor of the MDM2-p53 interaction and results in inhibition of p53 degradation by MDM2 and ultimately p53 accumulation/stabilisation (Vassilev *et al.*, 2004; Vassilev, 2007). **Etoposide** and **doxorubicin** are topo-isomerase II inhibitors. Topo-isomerase II is an enzyme that manages tangles and supercoils in the DNA by causing transient double strand breaks. After strand passage, topo-isomerase II relegates cleaved DNA (Hande, 2008). Etoposide and cisplatin inhibit this enzyme and cause double strand breaks (IR cause double strand breaks, too) (Santivasi and Xia, 2014). **Cisplatin**, and **UV** interfere with DNA replication by inducing DNA breaks which are effective blocks to RNA polymerase II (Mello, Lippard and Essigmann, 1995; Rastogi *et al.*, 2010). The resulting DNA damage leads to activation of p53 pathway as discussed in section **1.3.3**. The effect of p53 was then determined on the expression of either endogenous PLK1 levels or on a *PLK1* promoter which had been fused upstream of the luciferase gene in the pGL3 vector.

3.2 Aims

The aim of this chapter is to reassess mechanism(s) of repression of *PLK1* by p53, in light of new knowledge/model recently proposed.

3.3 Results

3.3.1 p53 induced by Nutlin causes repression of *PLK1*

To estimate the effect of p53 on *PLK1* repression, HCT116 (wild type, p53+/+) cells were treated with different p53 stabilising agents (these agents indeed increase the steady state levels of p53, but since they interfere with p53 degradation by MDM2, they are referred to as p53 stabilising agents). Nutlin as explained before, is an inhibitor of MDM2 and stabilises p53 by preventing its degradation by MDM2 (Vassilev *et al.*, 2004). Increasing concentrations of Nutlin cause a dose dependent stabilisation of p53 which is associated with reduction in PLK1 protein levels (**Figure 3-2**). These data suggest that p53 could be involved in PLK1 downregulation as increasing concentrations of Nutlin causes decreasing levels of PLK1. Interestingly the MDM2 levels increases dramatically with Nulin treatment, however less increase in MDM2 levels are observed when cells are treated with etoposide (whereas the p53 levels goes up dramatically in both Nutlin and etoposide treatment).

To further confirm that this effect is due to presence of p53, HCT116 p53-/- cells (an isogenic line which lacks full length p53) were treated with p53 stabilising agents. The data in **Figure 3-2** clearly show that treatment of these cells with Nutlin does not change the PLK1 levels. On the other hand, looking back at the HCT116 p53+/+ results, treating the cells with DNA damaging agent etoposide, increases the levels of p53, but interestingly there seem to be no reduction in the PLK1 levels (**Figure 3-2**).

To minimize the chances that the phenomenon we observe is specific to one cell line only, similar experiments were performed with U2OS cells that have wild type p53. In U2OS cells, also, treatment with Nutlin increases the p53 levels and downregulates PLK1 levels. However, p53 induced by etoposide and doxorubicin treatment resulted in a small reduction of PLK1 level and cisplatin and UV treatment did not cause any reduction in PLK1 **(Figure 3-3 A)**. Similar to HCT116 p53-/- cells, silencing p53 in U2OS cells prevented the downregulation of PLK1 by p53 inducing agents **(Figure 3-3 B)**.

Looking back at the data in **Figure 3-3 A**, all the p53 stabilising agents seem to cause an increase in p21 levels, however there is no increase in p21 when cells treated with doxorubicin. It is particularly interesting because induction of p53 by doxorubicin is higher/equal to p53 induction by other DNA damaging agents (etoposide, cisplatin and UV).

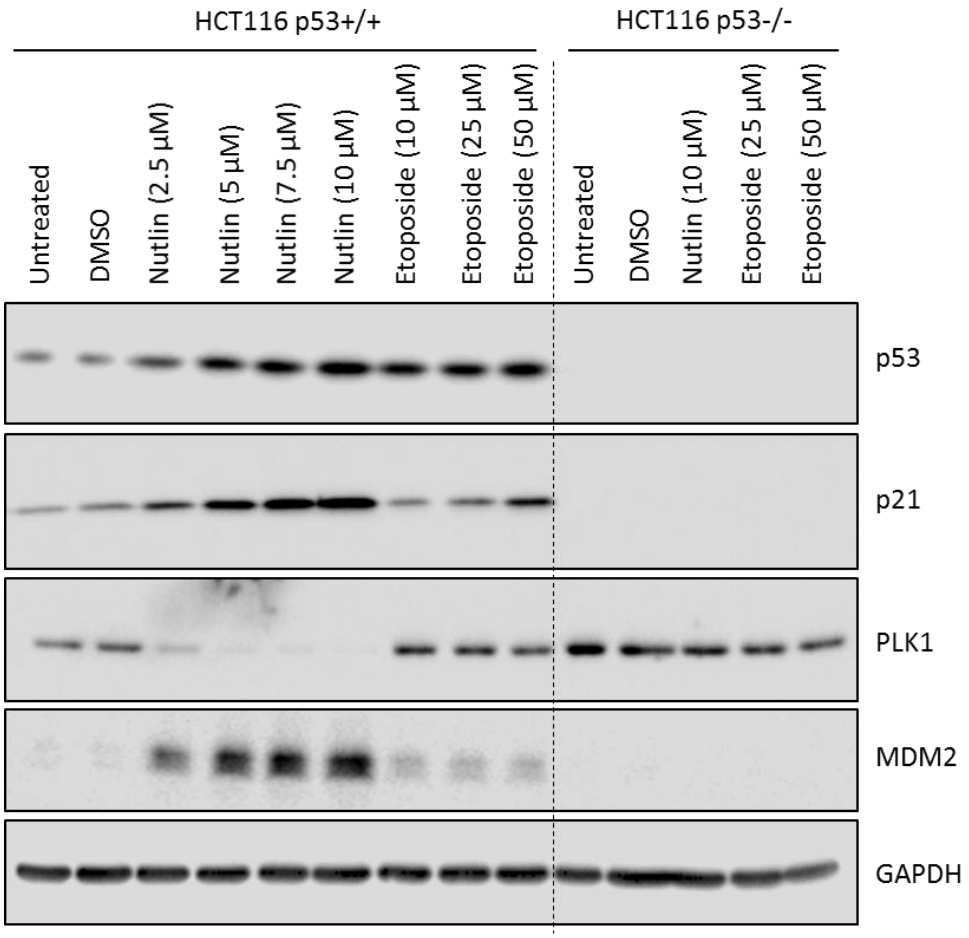


Figure 3-2. p53 induced by Nutlin, but not etoposide, downregulates PLK1 in HCT116 p53+/+ cells.
HCT116 cells were treated with increasing concentrations of Nutlin and etoposide for 24 hours. Cells were left untreated or treated with DMSO (vehicle) as control. Cell lysates were then used to perform western blot using antibodies indicated in the figure. HCT116 p53-/- cells were used as a control. These western blots are representative of 2 independent experiments.

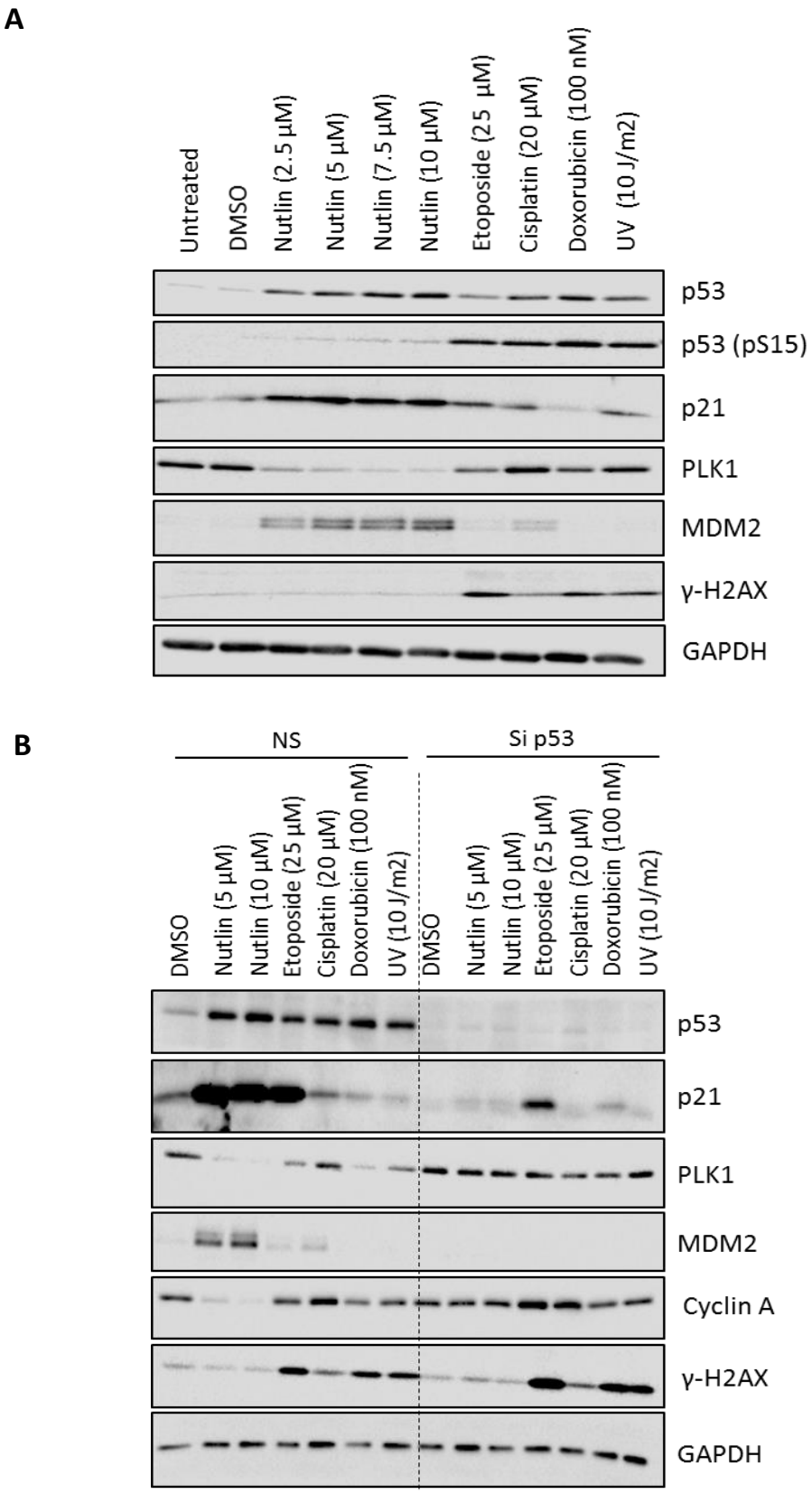


Figure 3-3. Downregulation of PLK1 in U2OS cells in response to p53 induction by different agents.
(A) U2OS cells were treated with increasing concentrations of Nutlin and DNA damaging agents for 24 hours. **(B)** p53 was silenced (si p53) or mock silenced (NS) in U2OS cells followed by 24 hours treatment with different p53 stabilising agents. Untreated and/or DMSO (vehicle) treated cells were used as control. Cell lysates were then prepared to perform western blot using antibodies indicated in the figure. These western blots are representative of 2 independent experiments.

3.3.2 Nutlin mainly induces G1 arrest whereas DNA damaging agents induce S or G2/M arrest

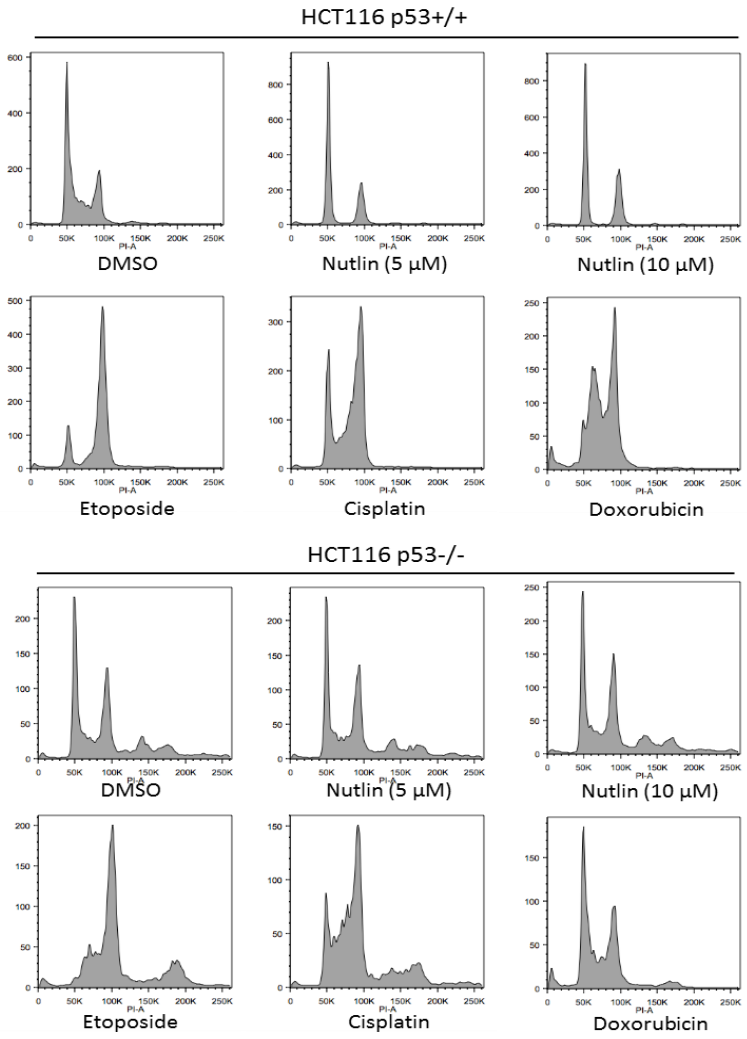
We were then interested to see why there are differences between the responses seen by Nutlin and DNA damaging agents, and how such differences could be explained mechanistically. As PLK1 is a cell cycle dependent protein and its levels are regulated over the course of the cell cycle, the first question asked was whether different p53 stabilising agents lead to different cell cycle profiles and that could lead to the observed differences.

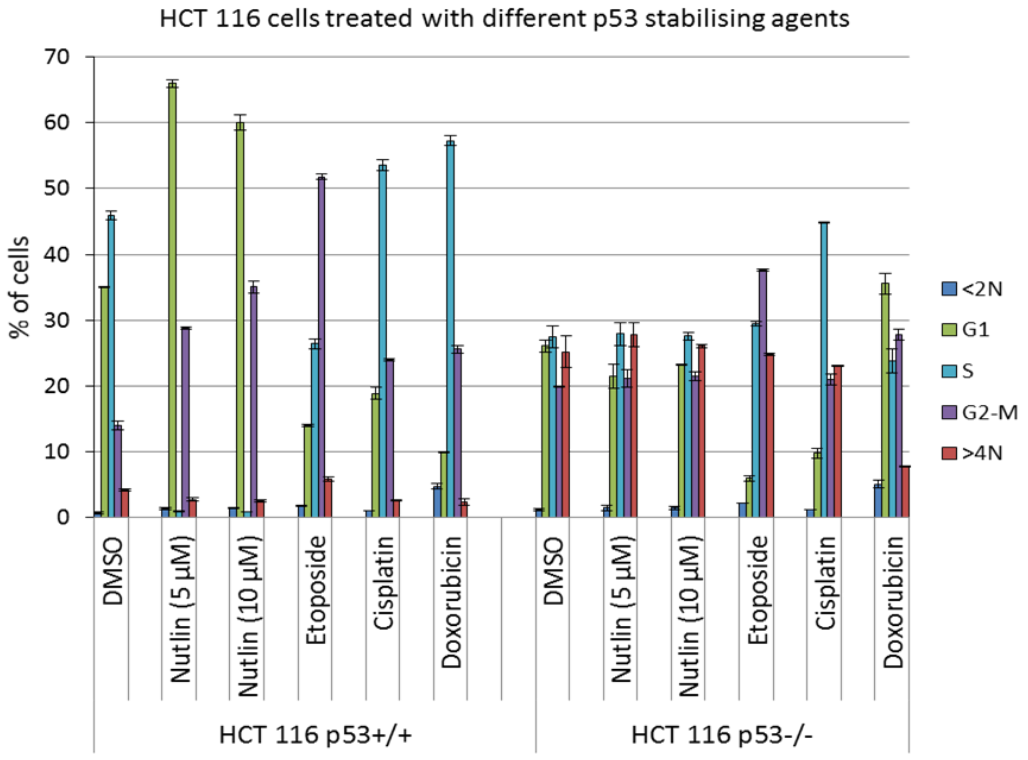
Flow cytometry was carried out after labelling drug treated HCT 116 and U2OS cells with BrdU and propidium iodide. Analysis of the data showed that Nutlin mainly causes arrest in G1 whereas etoposide causes arrest predominantly in G2 phase. Cisplatin and doxorubicin on the other hand resulted in higher population of cells in S phase **(Figure 3-4 A, B)**. Similar analysis in HCT116 p53-/- cells were carried out. The data showed that these effects are mainly dependent on p53 as most of the cell cycle arrest disappeared in p53-/- cells **(Figure 3-4 A)**. Therefore, one possible explanation for differences in PLK1 levels after treatment with different p53 stabilising agents could be that different drugs cause arrest in different stages of the cell cycle and the levels of PLK1 repression could be a reflection of the level of expression relative to its normal level at the given phase of the cell cycle.

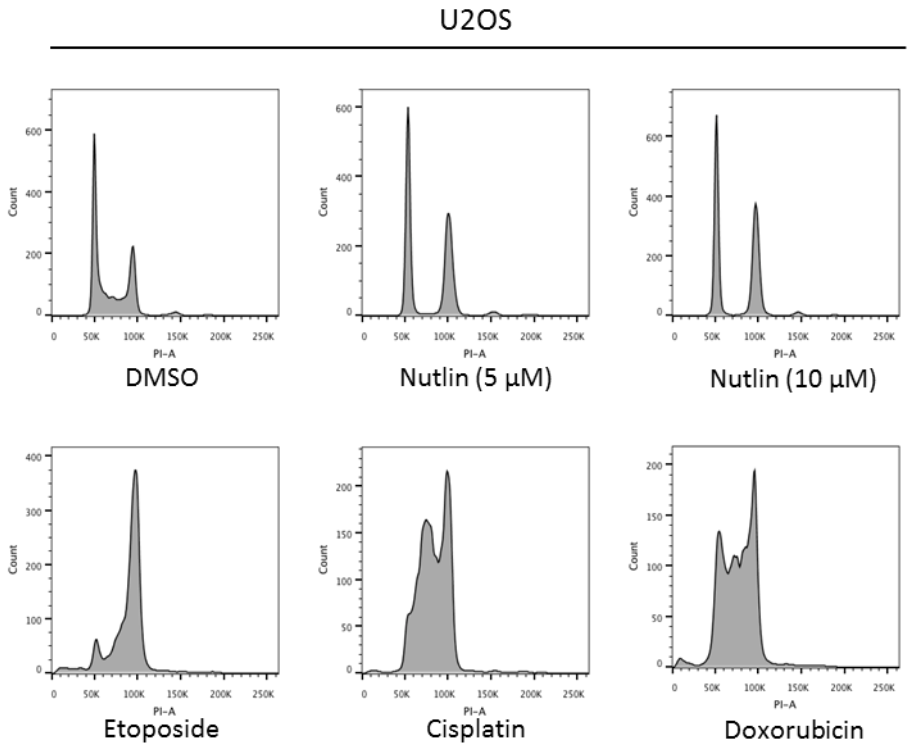
So, we proposed that Nutlin dissociates MDM2 and p53 interaction, as a result p53 gets stabilised and in turn induces p21 expression which mediates G1 arrest, at which stage the levels of PLK1 are low. On the other hand, DNA damaging agents stabilise p53 after causing damage to DNA which then arrest

the cells in S or G2 phase, phases of the cell cycle in which PLK1 levels are higher (**Figure 3-5**).

A


HCT116 p53+/+
DMSO
Nutlin (5 µM)
Nutlin (10 µM)
Etoposide
Cisplatin
Doxorubicin
HCT116 p53-/-
DMSO
Nutlin (5 µM)
Nutlin (10 µM)
Etoposide
Cisplatin
Doxorubicin


HCT 116 cells treated with different p53 stabilising agents
% of cells
<2N
G1
S
G2-M
>4N
DMSO
Nutlin (5 µM)
Nutlin (10 µM)
Etoposide
Cisplatin
Doxorubicin
HCT 116 p53+/+
HCT 116 p53-/-



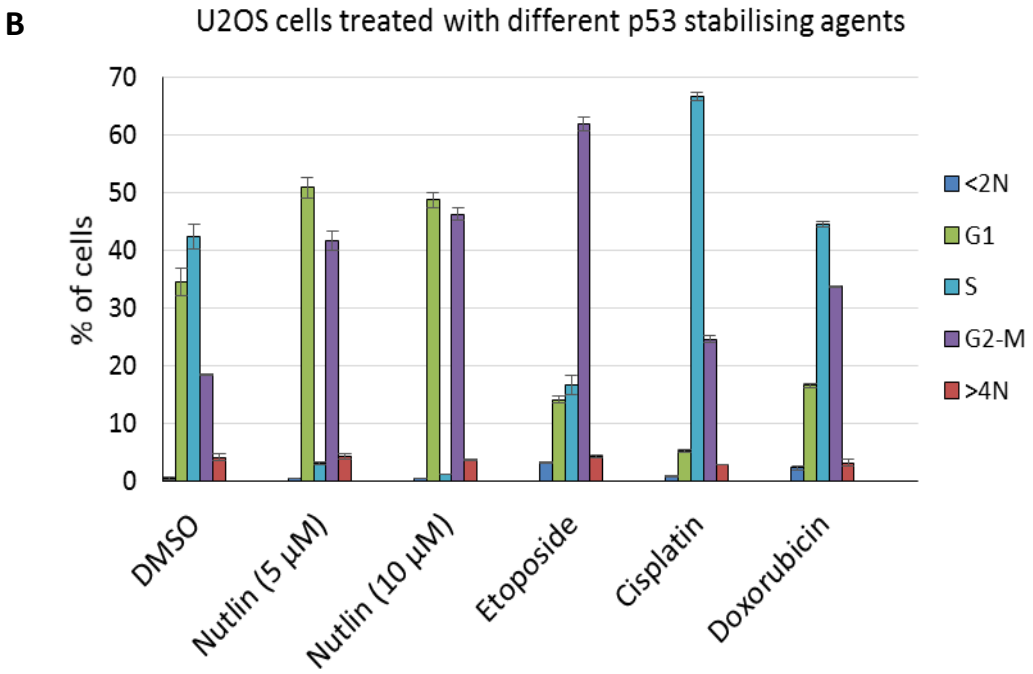


Figure 3-4. Different p53 stabilizing agents result in different cell cycle profile.
(A) HCT116 **(B)** U2OS cells were treated with Nutlin or DNA damaging agents (25 µM etoposide, 20 µM cisplatin, 100 nM doxorubicin) for 24 hours. Cells were then collected and labelled with propidium iodide and Brdu. Flow cytometry was carried out to estimate the population of cells in different phases of the cell cycle. Top panel are representative histograms and bottom panel is the quantification of the results. These results are representative of 2 independent experiments.

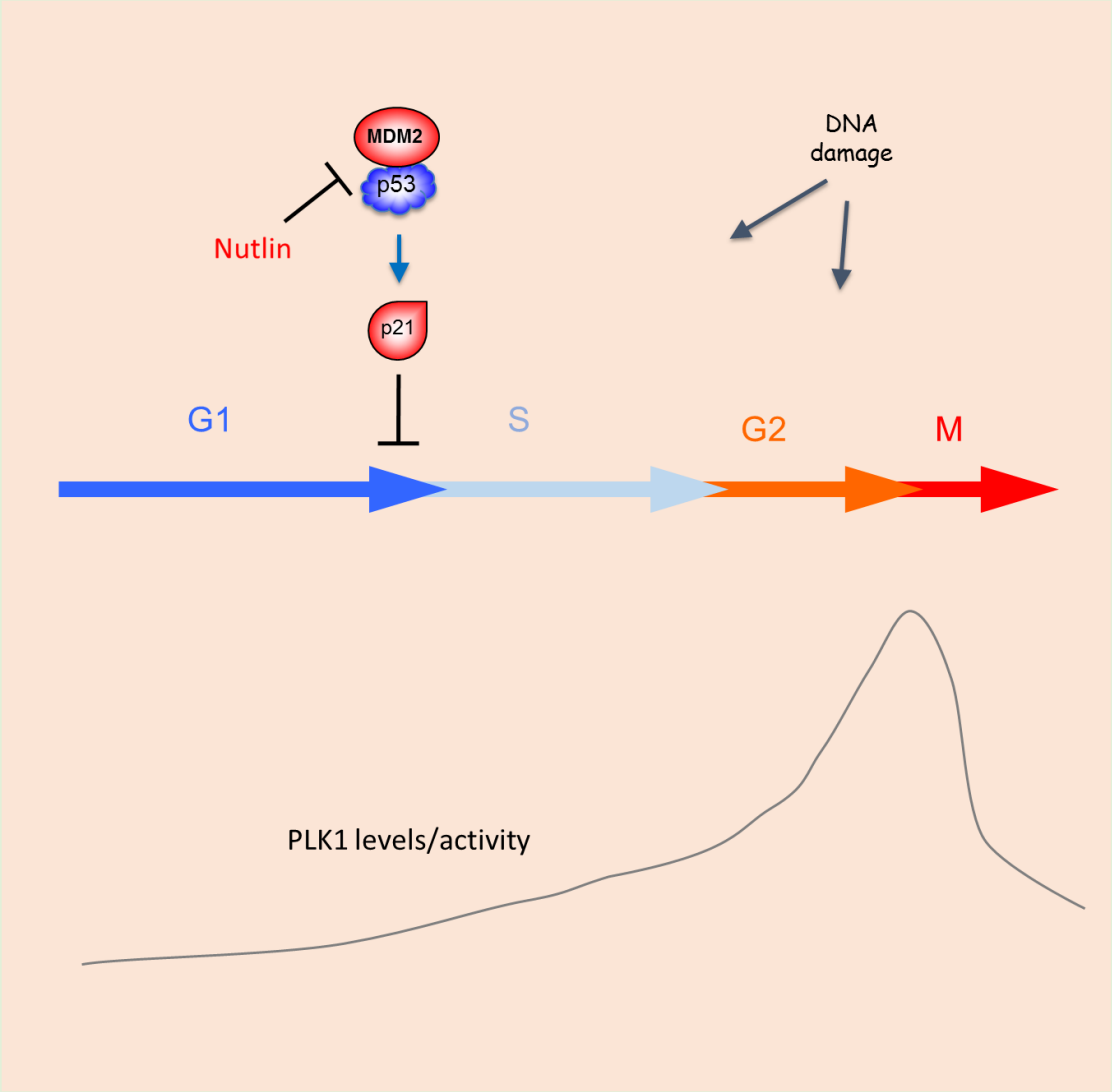


Figure 3-5. Schematic representation of proposed model for how Nutlin and DNA damaging agents might contribute to different behaviours in PLK1 repression by affecting cell cycle arrest.
Nutlin causes p53 stabilisation by inhibiting p53-MDM2 interaction. As the result, p53 induces p21 which is involved in G1 arrest, a stage of the cell cycle where PLK1 levels are low. DNA damaging agents, on the other hand, causes S and G2 arrest, where PLK1 levels are higher (the line graph is an estimation of PLK1 levels/activity and is not based on actual data).

3.3.3 Nutlin-induced accumulation of cells in G1 is p21 dependent and p21 knockdown can partly relieve the PLK1 downregulation

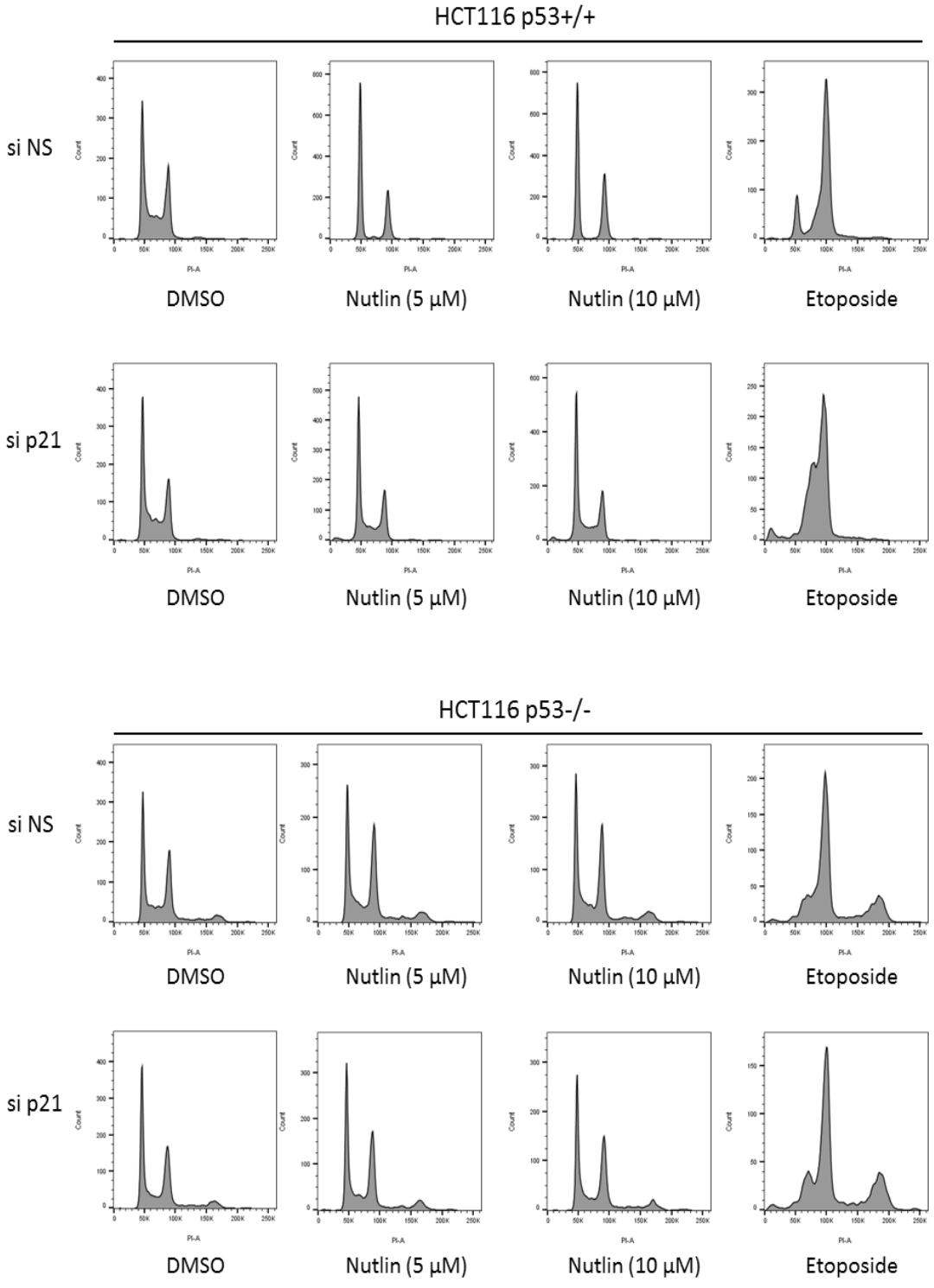
As p21 is a classic downstream target of p53 which is involved in G1 arrest, we were interested to check if the G1 peak that appears in cell cycle analysis of cells treated with Nutlin (**Figure 3-4**) is due to induction of p21 by p53. To check this possibility p21 was silenced with p21 siRNA. Cell cycle analysis showed that when p21 is silenced, the G1 peak induced by Nutlin greatly reduces **(Figure 3-6)**. So, the curious question then was what will happen at PLK1 levels in cells treated with Nutlin when p21 is silenced?

To address this, p21 was silenced in the HCT116 and U2OS cells. The cells were then treated with Nutlin or DNA damaging agents. Western blot analysis showed that part of the PLK1 repression caused by induction of p53 is rescued in cells with silenced p21 compared to control cells expressing non-silencing siRNA **(Figure 3-7 A, B)**. These data further support our hypothesis that p53 mediated repression of PLK1 as determined/perceived from western blots could reflect cell cycle periodicity i.e. where in the cell cycle most of the population of the cells are at the time of analysing them. Also given the importance of p21 in PLK1 repression, as indicated by **Figure 3-7 A, B,** the data support the idea that the p21-DREAM-CDE/CHR model could be the major effector of PLK1 repression by p53. Complete rescue of the PLK1 downregulation with p21 siRNA is not observed either because p21 knockdown did not eliminate p21 completely or because there might be additional/alternative mechanisms involved.

To confirm the results of siRNA and eliminate the possibility that the residual p21 levels after p21 knockdown could have caused the small PLK1 repression,

we also used HCT116 p21-/- cells which are p21 null. Western blot analysis of HCT116 p21-/- cells showed that, similar to p21 knockdown results, downregulation of PLK1 in the Nutlin treated cells is still detectable **(Figure 3-7 C)**. However, since in HCT116 p21-/- cells treated with Nutlin the downregulation of PLK1 is very small as compared to HCT116 p53+/+ cells, it would have been helpful to quantify the data (by densitometry) to obtain the exact fold differences.

A


HCT116 p53+/+
si NS
DMSO
Nutlin (5 µM)
Nutlin (10 µM)
Etoposide
si p21
DMSO
Nutlin (5 µM)
Nutlin (10 µM)
Etoposide
HCT116 p53-/-
si NS
DMSO
Nutlin (5 µM)
Nutlin (10 µM)
Etoposide
si p21
DMSO
Nutlin (5 µM)
Nutlin (10 µM)
Etoposide

B

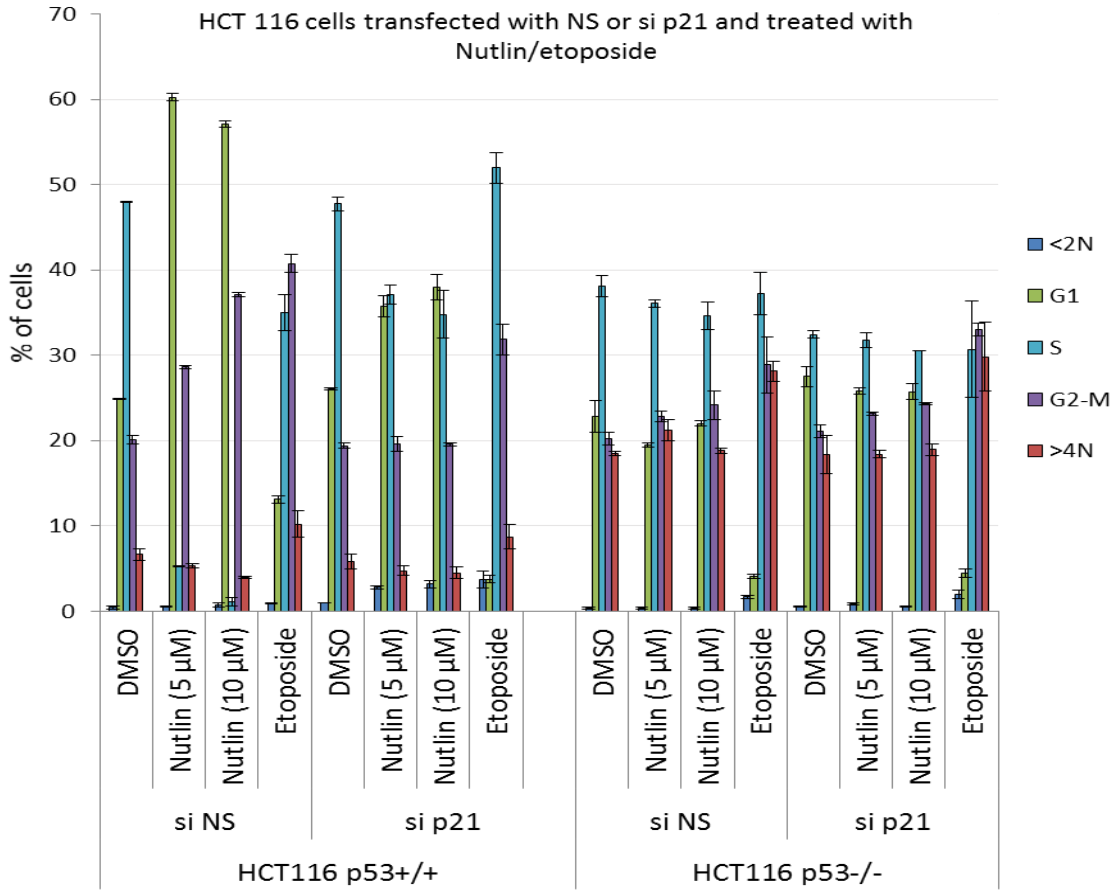


Figure 3-6. p21 contributes to peak G1 population in cells treated with Nutlin.
HCT 116 cells transfected either with NS siRNA or si p21 and then treated with 5 and 10 µM Nutlin or 25 µM etoposide. Cells collected after 24 hours of treatment and labelled with propidium iodide and Brdu. Cell cycle analysis by flow cytometry carried out. **(A)** Histograms, **(B)** quantification of the results in A. These results are representative of 2 independent experiments.

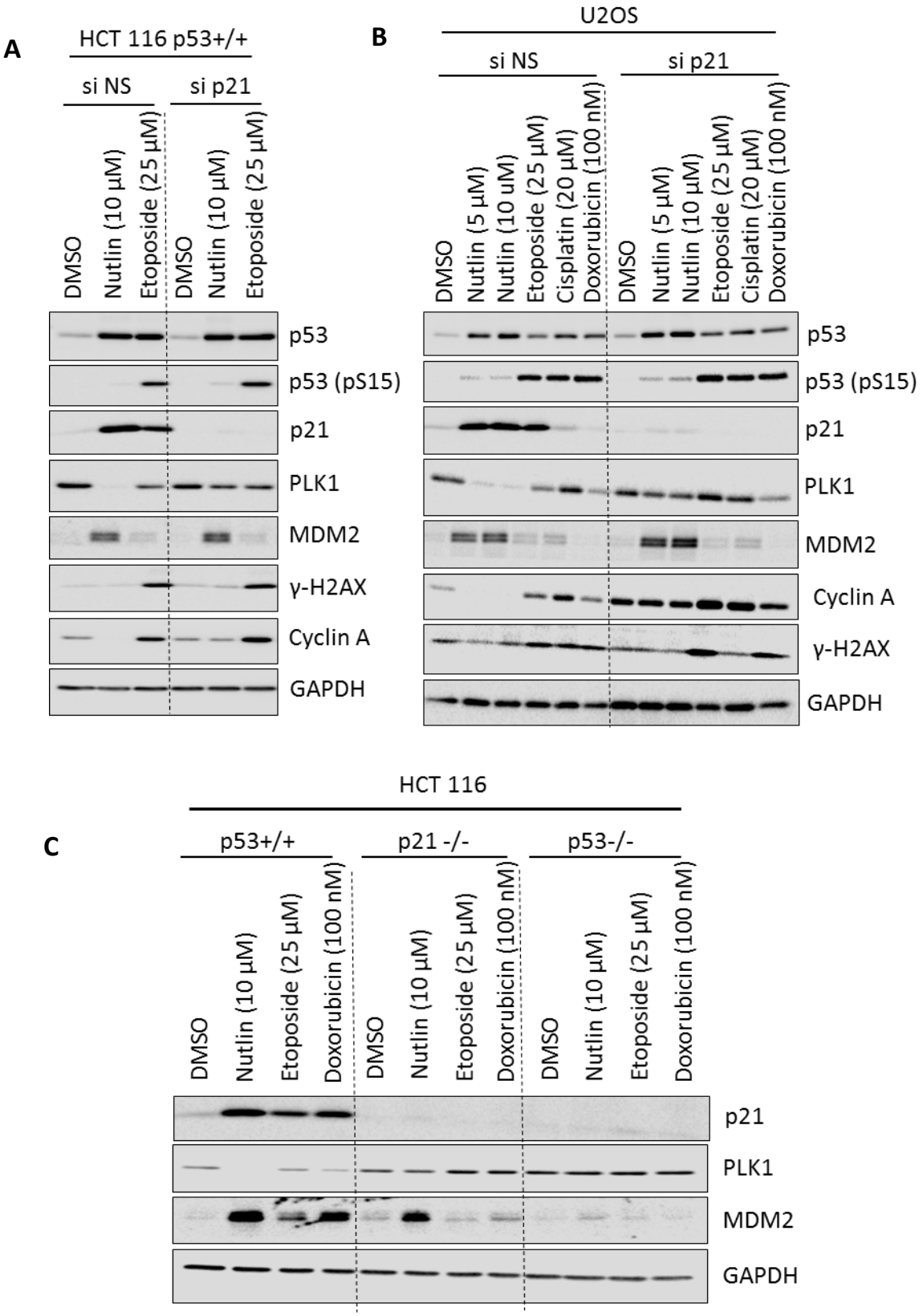


Figure 3-7. p21 knockdown can partly relieve the downregulation of PLK1 by p53.
(A) HCT116 or **(B)** U2OS cells were transfected with si NS or si p21. Cells were then treated with different drugs for 24 hours after which lysates were prepared, and western blotting was carried out for various proteins indicated in the figure. **(C)** HCT 116 p53+/+ and its p21 null (p21-/-) and full length p53 null (p53-/-) derivatives were treated with DMSO (vehicle, as control), Nutlin, etoposide or doxorubicin for 24 hours. Cells were then lysed and proceeded for western blotting with antibodies indicated in the figure. These western blots are representative of 2 independent experiments.

3.3.4 The repression of PLK1 by p53 appears to occur by both p21 dependent and p21 independent mechanisms

To estimate the effect of p21 on PLK1 at the level of promoter activity, we used a pGL3 plasmid in which *PLK1* promoter was fused upstream of luciferase gene, so luciferase could express under the control of *PLK1* promoter. p21 was silenced in H1299 cells (which are p53 null) and *PLK1* promoter activity was compared with control (mock silencing) after transfecting cells with p53. Luciferase reporter assays were performed and the results showed that there is a strong repression in *PLK1* promoter activity upon p53 addition in both p21 silenced cells and the control, however the reduction is less where p21 is silenced **(Figure 3-8 A)**. These data suggest that p21 is important in PLK1 repression by p53, but p53 can still repress PLK1 promoter in the absence of p21.

Also, to further investigate how p21 and p53 can influence *PLK1* promoter activity, H1299 cells ectopically expressing elevated p21 or p53 (along with *PLK1* promoter fused to luciferase gene in a pGL3 plasmid) were analysed by luciferase reporter assay. The data suggest that p53 and p21 both cause reduction of *PLK1* promoter activity. Cells in which both p21 and p53 were ectopically expressed, result in additive effect **(Figure 3-8 B).** These results show that there might be a p21 independent mechanism as well.

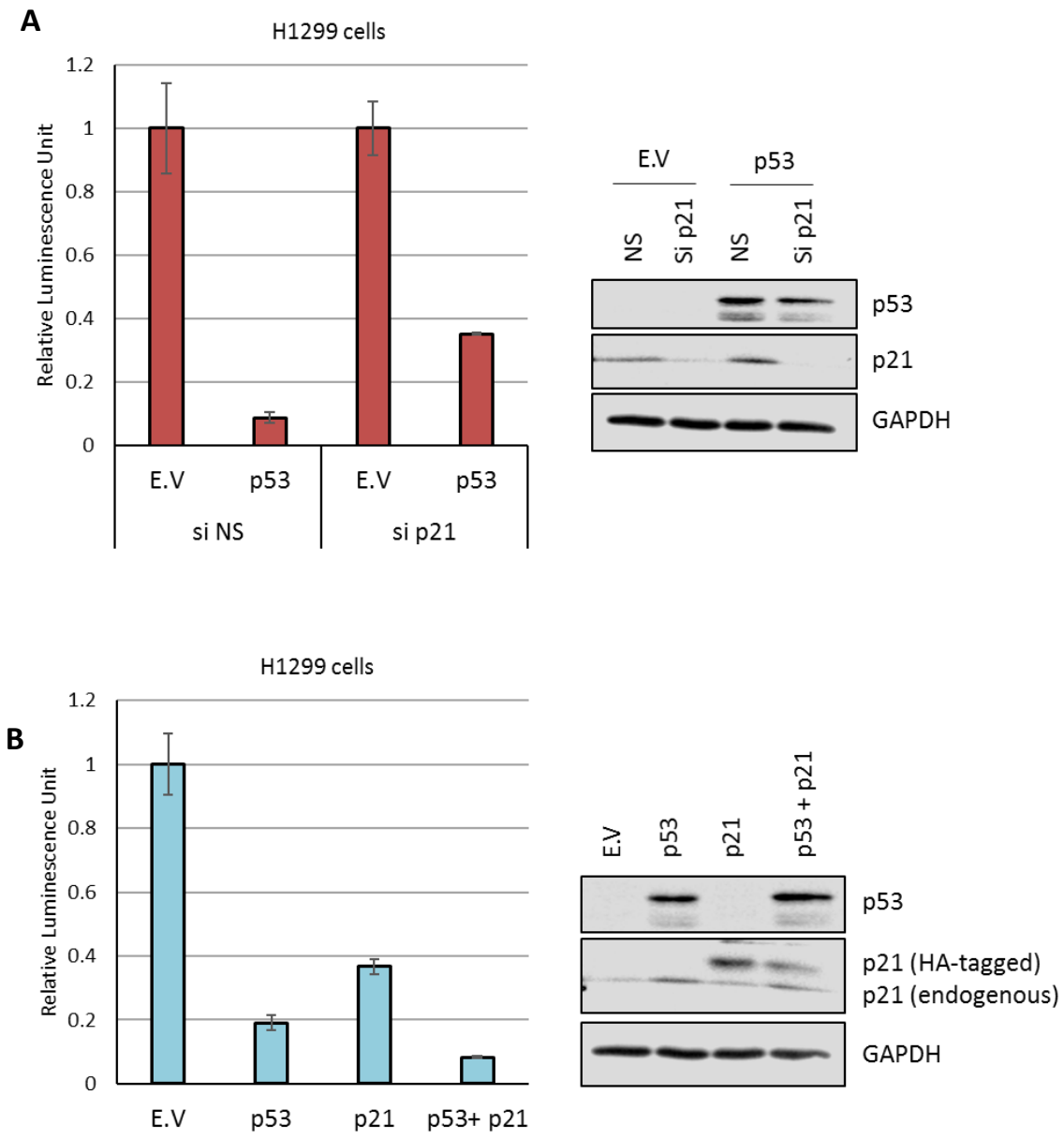


Figure 3-8. p21 is important for p53 mediated *PLK1* repression but it appears that a p21 independent mechanism could be involved too.

(A) H1299 cells were p21 silenced (si p21) or mock silenced (si NS) after which transfected with *PLK1* promoter along with either p53 or empty vector (E.V). Equal amounts of protein was used for each reaction. Luciferase assay was then carried out. **(B)** H1299 cells transfected with *PLK1* promoter along with plasmids containing p53 (10 ng) or p21 (50 ng) or a combination of both plasmids. Empty vector (E.V) was used as a control. *PLK1* promoter activity was then measured by luciferase assay (the experiment is normalised for protein not for transfection efficiency). The results are shown relative to empty vector (E.V). Western blotting was then performed with the samples from the luciferase assays to check the level of transfection. These results are the representative of at least 2 independent experiments.

3.3.5 Direct or indirect PLK1 repression by p53 could be cell line dependent

As previously mentioned (section 1.2.5), PLK1 is a cell cycle dependent protein and its levels change as cells proceed through the cycle. This regulation is mediated by CDE/CHR elements in the promoter. With the data so far, we know that p21 could be important for p53 mediated repression of PLK1, but we don't know whether this is simply through DREAM complex or other mechanisms could be involved too.

To address this question, we first transiently transfected HCT116 p53+/+ and U2OS cells with plasmids containing *PLK1* promoter (wild type or CDE/CHR mutated) fused to a firefly luciferase as a read out (see materials and methods **Figure 2-1**). An empty vector was used as a control. Transfected cells were then treated with Nutlin or etoposide to induce p53. Luciferase assays were performed to check the *PLK1* promoter activity in these cells. **Figure 3-9** shows that treatment with Nutlin caused repression of the wild type *PLK1* promoter activity in both cell lines. Treatment with etoposide on the other hand showed apparent increase in HCT 116 and no/little (non-significant) changes in U2OS cells (similar to endogenous PLK1 protein levels showed in **Figure 3-2** and **Figure 3-3,** suggesting that the reporter assays mirror physiological changes in PLK1 repression). Analysis of the CDE/CHR mutant PLK1 promoter showed loss of responsiveness in HCT 116 cells and partial loss in U2OS cells (**Figure 3-9)**. These data suggest that in HCT116 cells, CDE/CHR elements seem to play an important role in the repression mechanism, whereas U2OS cells seem to be able to still repress *PLK1* promoter in absence of cell cycle regulatory element (CDE/CHR) but only in part.

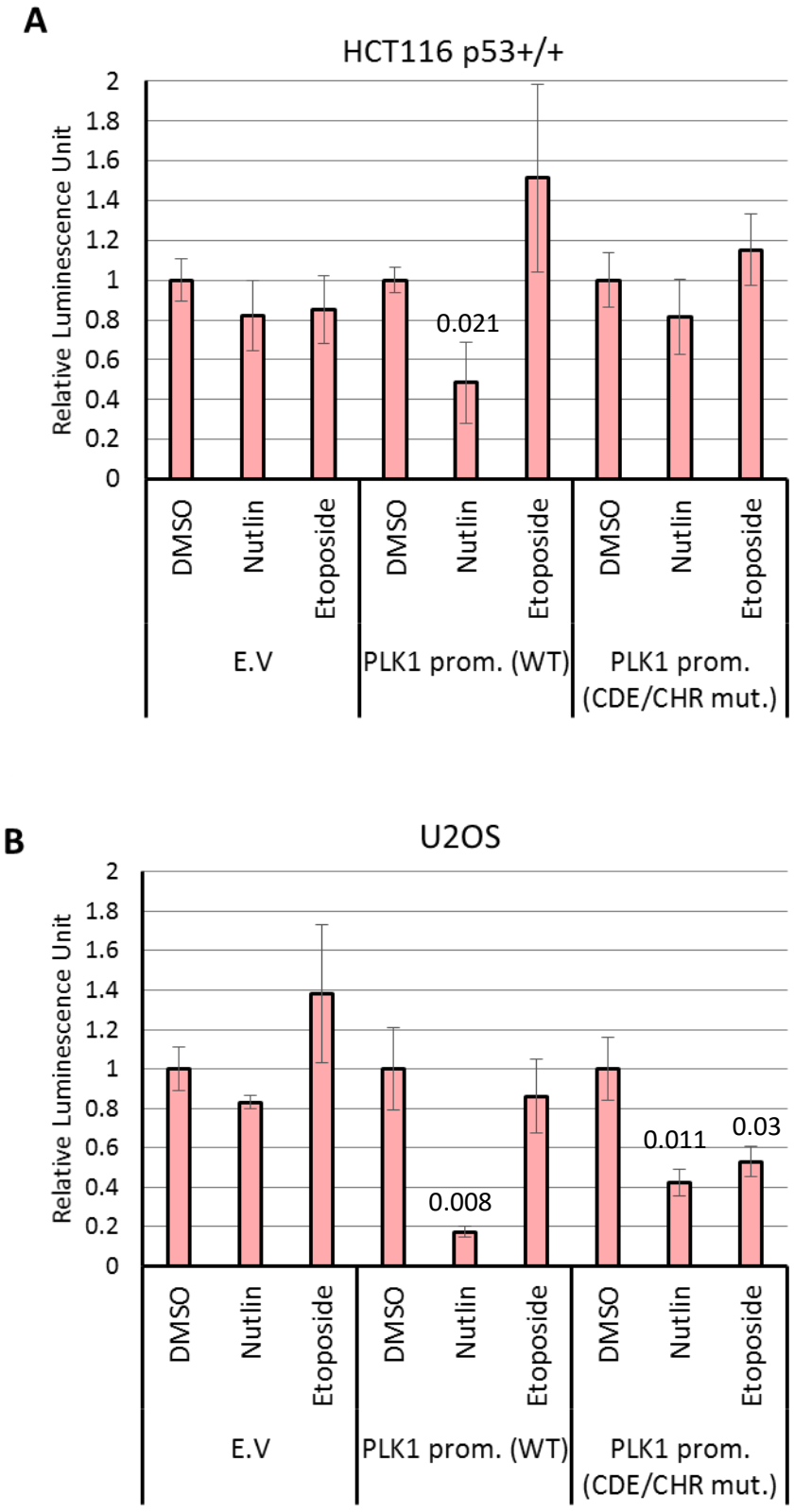
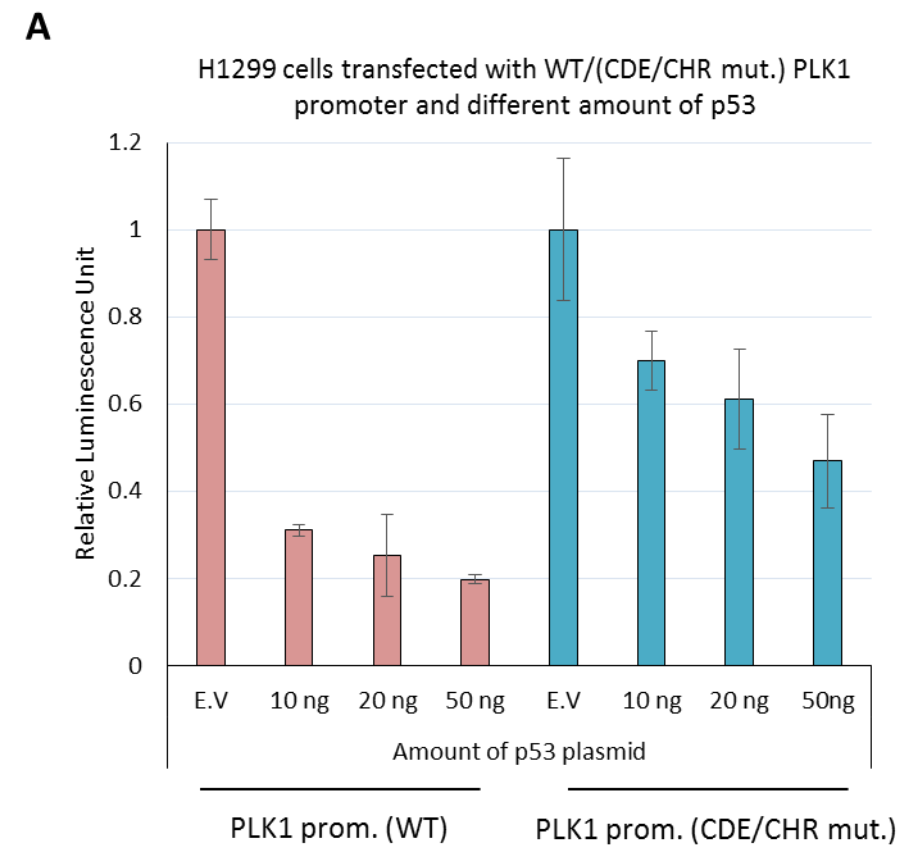


Figure 3-9. Direct or indirect *PLK1* repression by p53 could be cell line dependent.
(A) HCT116 p53+/+, **(B)** U2OS cells transfected with empty vector (containing only the luciferase gene and not PLK1 promoter)/wild type PLK1 promoter/PLK1 promoter mutated at CDE/CHR element. Transfected cells were then treated with 10 µM Nutlin or 25 µM etoposide or DMSO (vehicle) as a control. After 24 hours of treatment samples were harvested and luciferase assay was carried out. These results are the average of 3 independent experiments. The numbers on the bars refer to p-value as determined by t-test.

We also used p53 null H1299 cells to further investigate the matter. Cells transfected with wild type and mutant *PLK1* promoter plasmids and their performance were compared upon transfection with p53-expressing plasmid. Luciferase assay results showed that the repression of *PLK1* promoter (both in wild type and CDE/CHR mutant) is stimulated by p53 in a dose-dependent manner. However, H1299 cells transfected with Wild type *PLK1* promoter showed much sharper reduction of luciferase activity in response to p53 compared to PLK1 promoter that has the CDE/CHR mutation **(Figure 3-10).** All together these data suggest a cell line dependency on CDE/CHR element in *PLK1* repression by p53.



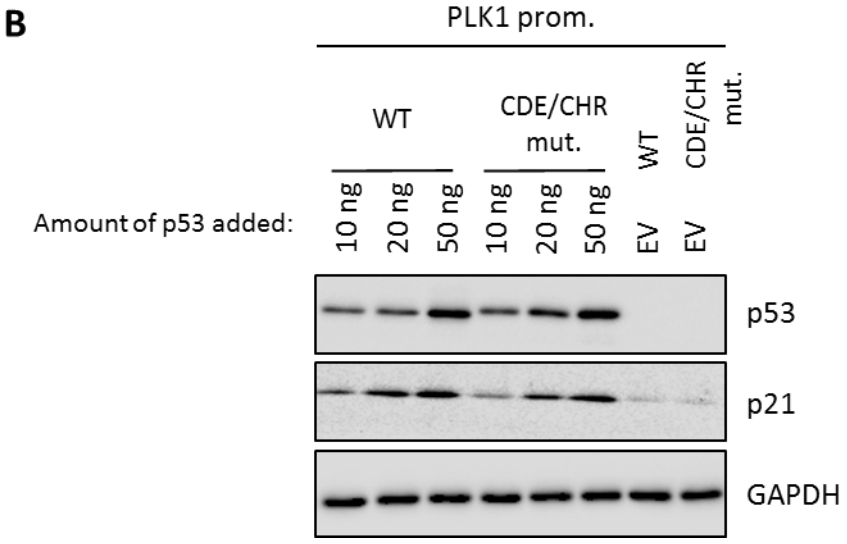


Figure 3-10. p53 induced *PLK1* repression in H1299 cells partly depends on CDE/CHR elements. **(A)** H1299 cells transfected with either wild type or CDE/CHR mutated PLK1 promoter in combination with different amounts of p53 expressing plasmid. Empty vector (E.V, 0 ng of p53) was used as control. Luciferase assay was then carried out. **(B)** Western blot analysis was performed using the samples in figure A to check the transfection efficiency. These results are the average/representative of at least 2 independent experiments.

3.3.6 Cells stably transfected with *PLK1* promoter mutated at CDE/CHR elements show constant expression of luciferase at different stages of the cell cycle

To further confirm the data and eliminate any possible effect of transient transfection results might not be accurate, we generated the cells which stably express luciferase under control of *PLK1* promoter (see section 2.4.9). By doing so we expect that cells harbouring plasmid containing wild type *PLK1* promoter should express low luciferase levels/activity (which measures *PLK1* promoter activity) at early stages of the cell cycle and increased/elevated expression at later stages (i.e., they should mirror endogenous PLK1 expression). Whereas expression of luciferase in cells with *PLK1* promoter mutated at CDE/CHR elements is expected to be constant throughout the cell cycle. The effect of p53 on expression of *PLK1* in these cells (CDE/CHR mutant) then would be independent of CDE/CHR.

To test this, cells were first synchronised by serum starvation for 24 hours. They were then collected after 0, 4, 12, 16 and 24 hours following replacement of serum free media with media containing 10% FBS. Luciferase reporter assay was then carried out. **Figure 3-11 A** shows that after re-treating the cells with media containing serum, which should allow the cells to re-enter cell cycle and progress synchronously, *PLK1* promoter activity increases over time in clones with wild type *PLK1* promoter. Whereas in clones with *PLK1* promoter mutated at CDE/CHR elements constant *PLK1* promoter activity is observed in different time points. Western blot analysis also confirmed that Luciferase levels increase over time only in cells having wild type *PLK1* promoter and not in *PLK1* promoter with CDE/CHR mutation. Endogenous *PLK1* levels however increase

in both cell lines containing wild type and mutated *PLK1* promoter **(Figure 3-11 B).**

Here it would have been helpful to confirm the cell cycle profile of our samples at different time points collected. Cell cycle analysis by flow cytometry or even western blot analysis using antibodies against Rb or different cyclins would help us to determine whether cells have remained synchronised after re-stimulation by serum.

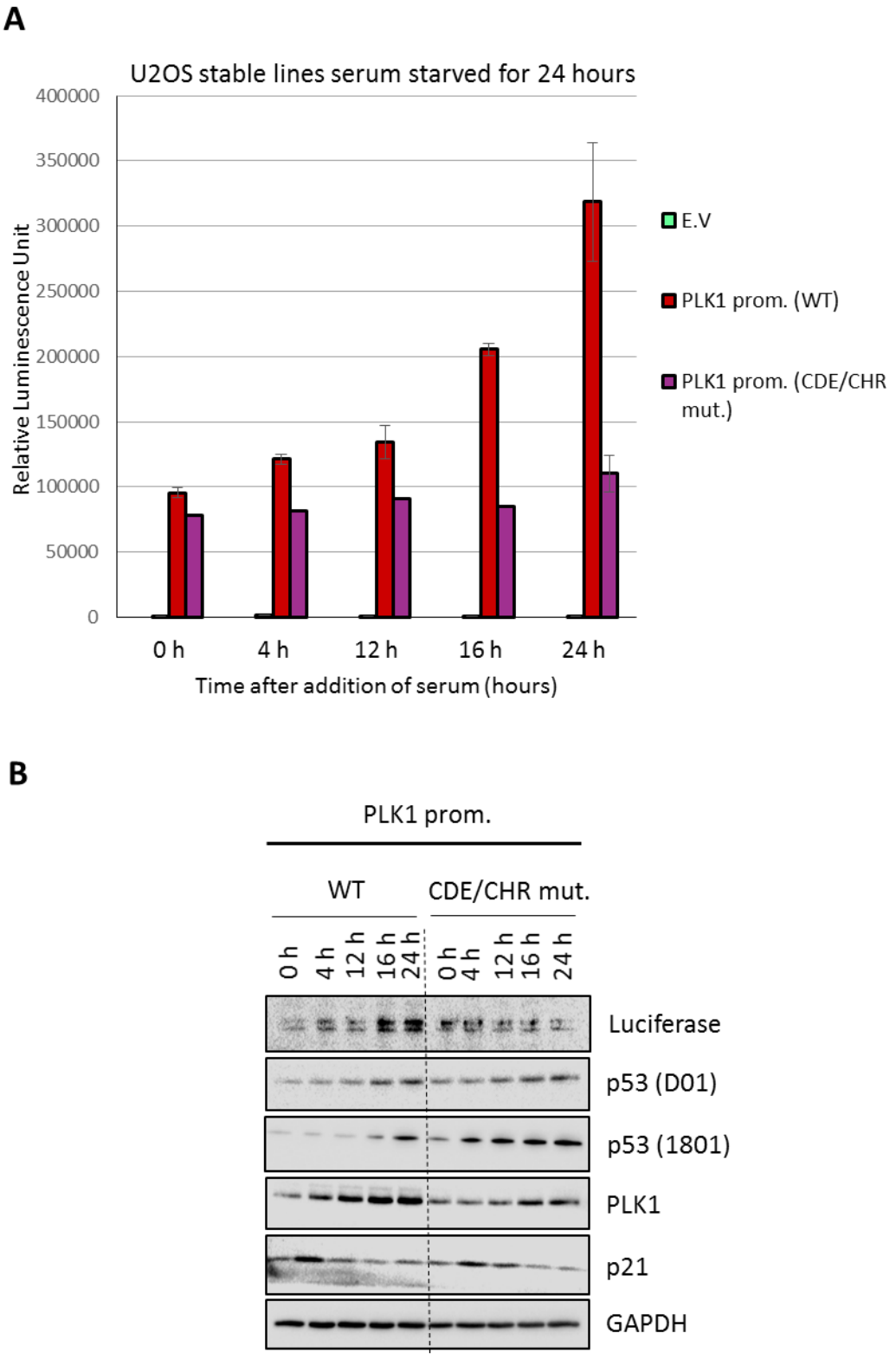


Figure 3-11. Cells stably transfected with *PLK1* promoter mutated at CDE/CHR elements show constant expression of luciferase at different stages of the cell cycle.

(A) U2OS cells stably express wild type *PLK1* promoter/*PLK1* promoter mutated at CDE/CHR elements/empty vector (use as control) fused to a luciferase reporter gene were synchronised by serum starvation for 24 hours. Lysates of cells collected at different time points after terminating serum starvation. Luciferase assays were then performed to measure *PLK1* promoter activity. **(B)** Western blot analysis performed by using the samples used in luciferase assay, for the proteins indicated in the figure. These results are representative of 2 independent experiments.

3.3.7 *PLK1* repression by Nutlin in U2OS cells is only partly dependent on CDE/CHR elements

Next, we treated the aforementioned stable cell lines with p53 stabilising agents and checked the *PLK1* promoter activity. In the cells that have the wild type *PLK1* promoter, the repression of *PLK1* promoter occurs upon treatment with Nutlin, etoposide and doxorubicin. Interestingly, the repression occurs with all 3 agents even in cells harbouring mutation in the CDE/CHR element **(Figure 3-12)**. These data demonstrate that in these cells p53 mediated repression of PLK1 could still happen without the functional CDE/CHR elements. **Figure 3-13 A and B** shows the results of two independent clones.

To confirm that the effect we observe is indeed due to p53, we silenced p53 by siRNA in the clones and then treated the cells with p53 stabilising agents. In cells harbouring wild type *PLK1* promoter, treating the cells with all 3 agents caused repression of the *PLK1* promoter when p53 was present, however in the p53 silenced cells, the repression was relieved, suggesting that p53 indeed is necessary for the repression mechanism. On the other hand, in cells harbouring mutated CDE/CHR element, the repression occurs in the control cells (non-silencing siRNA) upon treatment with all 3 agents, however silencing p53 completely abolishes the *PLK1* repression by Nutlin only, the repression by etoposide and doxorubicin partly relieves (**Figure 3-13**).

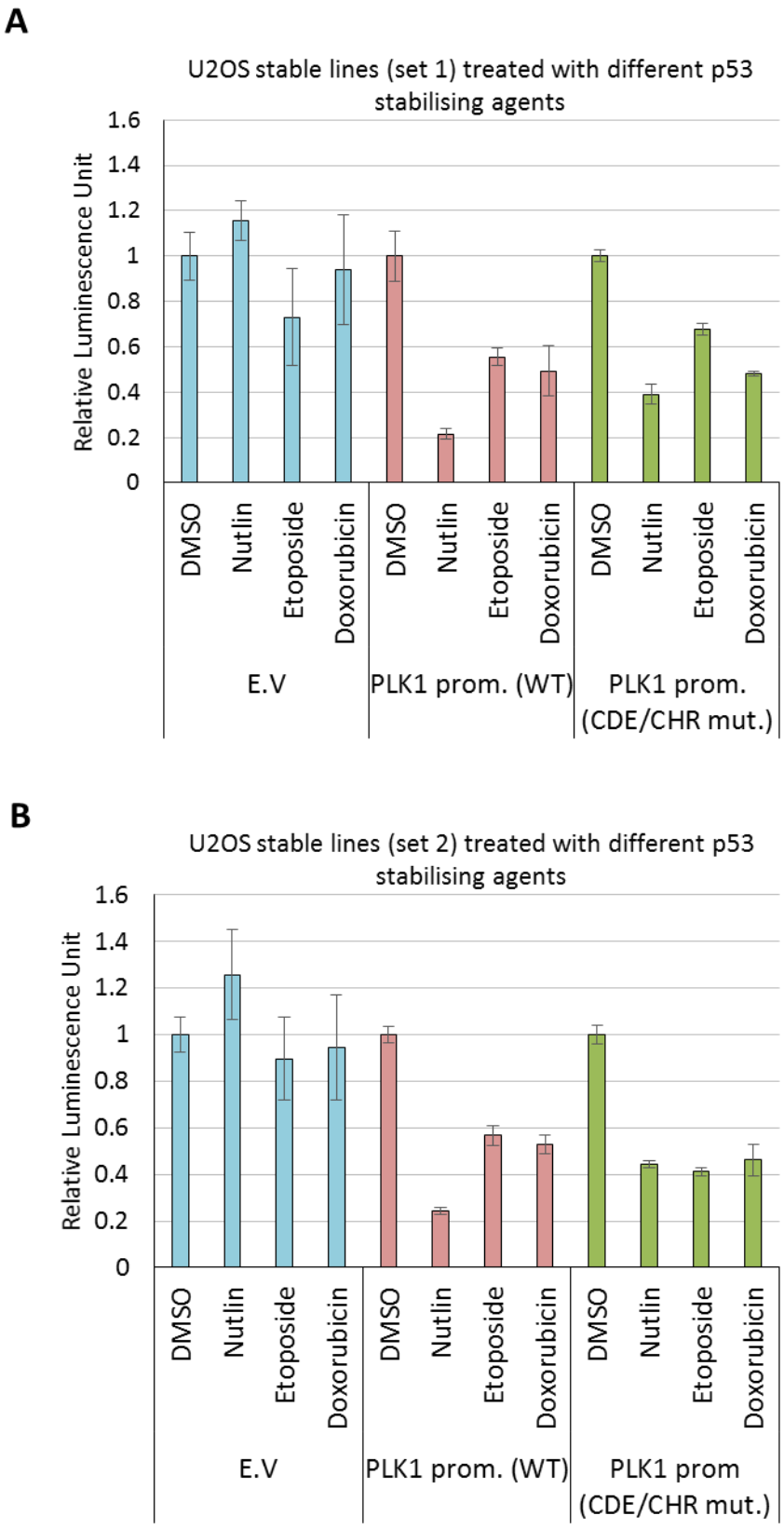


Figure 3-12. *PLK1* repression by p53 seems to be independent of CDE/CHR and cell cycle regulation in U2OS cells.

U2OS cells stably express wild type *PLK1* promoter/PLK1 promoter mutated at CDE/CHR elements/empty vector were treated with DMSO (as vehicle control), 10 µM Nutlin, 25 µM etoposide or 100 nM doxorubicin for 24 hours. Samples were then lysed and proceeded for luciferase assay analysis. **(A)** and **(B)** shows the results of 2 independent clones.

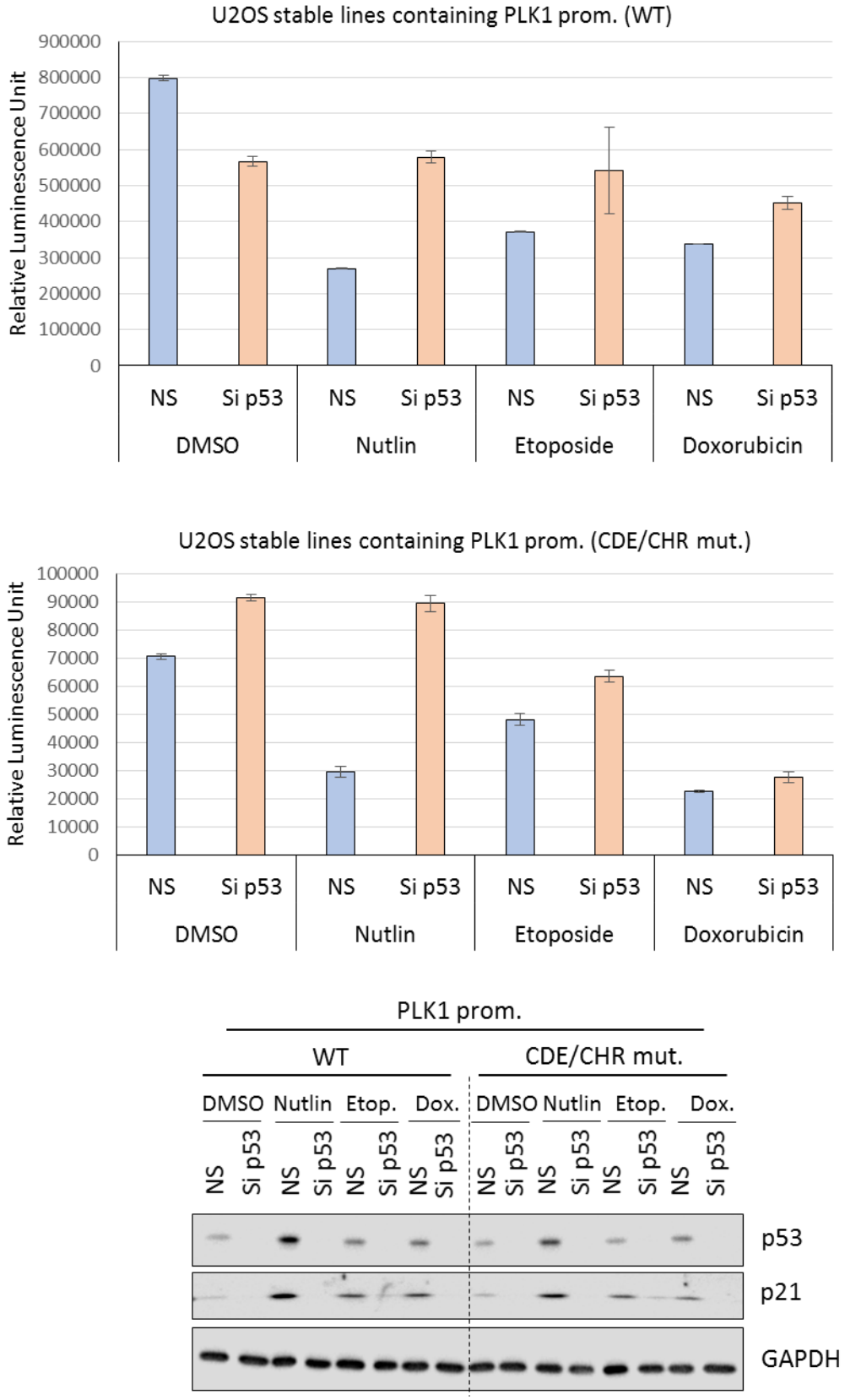


Figure 3-13. p53 dependency of *PLK1* repression induced by different p53 inducing agents.
(A) U2OS stable lines were transfected with si p53 or si NS (as control) and then treated with 10 µM Nutlin, 25 µM etoposide or 100 nM doxorubicin. DMSO (vehicle) treated cells were used as control. Luciferase assay was then performed. **(B)** Western blot analysis were carried out with the luciferase assay samples in figure A to check the knock down efficiency. These results are representative of 2 independent experiments.

3.3.8 The repression of *PLK1* by p53 may be more complex than regulation through the CDE/CHR elements alone

As there are conflicting results published in the literature regarding which part of *PLK1* promoter is required for its repression through p53, we wanted to see what will happen if we have deletions of the p53REs and mutation of CDE/CHR elements together; would p53 still cause repression of *PLK1* promoter?

Plasmids containing wild type *PLK1* promoter, *PLK1* promoter that has deletion in p53RE1, or deletions of both p53RE1 and p53RE2 upstream of luciferase gene in pGL3 plasmid (previously made in the laboratory, (Iyer *et al.*, 2014)) were used to introduce CDE/CHR mutations.

We used HCT116 p53+/+ and U2OS cells, transfected them with the above plasmids and then treated them with Nutlin to induce p53. Luciferase assays were then carried out. **Figure 3-14 A** shows that in HCT116 cells, there is *PLK1* repression upon Nutlin treatment with or without p53RE deletions, however this repression is not seen when CDE/CHR is mutated, further confirming the previous data that the *PLK1* repression by p53 in these cells is dependent on CDE/CHR elements.

Similarly, in U2OS cells there was very little difference in luciferase expression in wild type and p53REs deleted plasmid transfections. Also, mutation of the CDE/CHR elements had only minimal effect on relieving the repression (**Figure 3-14 B**), further confirming that in U2OS cells the repression of PLK1 by p53 is not solely dependent on CDE/CHR elements and other mechanisms could be involved.

We also used p53 null H1299 cells and transfected them with the different truncated and mutated *PLK1* promoter plasmids along with increasing levels of p53. Luciferase assay analysis showed that p53 causes repression of wild type *PLK1* promoter, but part of this repression is lost when CDE/CHR is mutated. Deletion of p53RE1 or p53RE1+2 has no/minimal effect on the repression. Similarly, when both CDE/CHR elements and the p53REs were mutated there was no significant additional loss of repression (**Figure 3-15**).

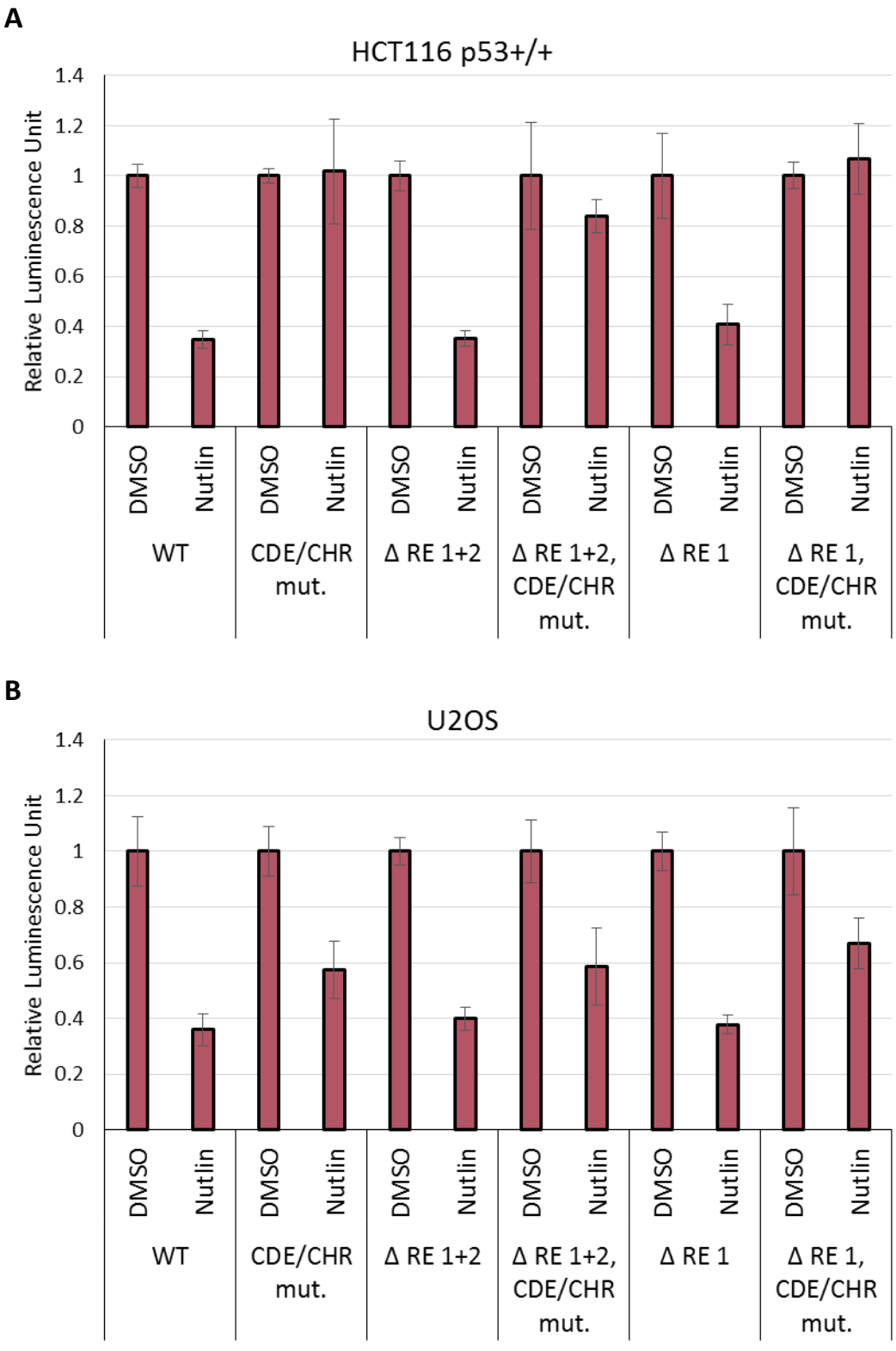


Figure 3-14. p53 responsive elements of *PLK1* promoter seem to have no significant role in *PLK1* repression by p53.

(A) HCT116 **(B)** U2OS cells were transfected with WT and different mutated versions of PLK1 promoter (as labelled in the figure). Transfected cells were then treated with Nutlin or DMSO (vehicle control) for 24 hours after which cells were lysed and luciferase assay was performed. These results are the average of at least 2 independent experiments.

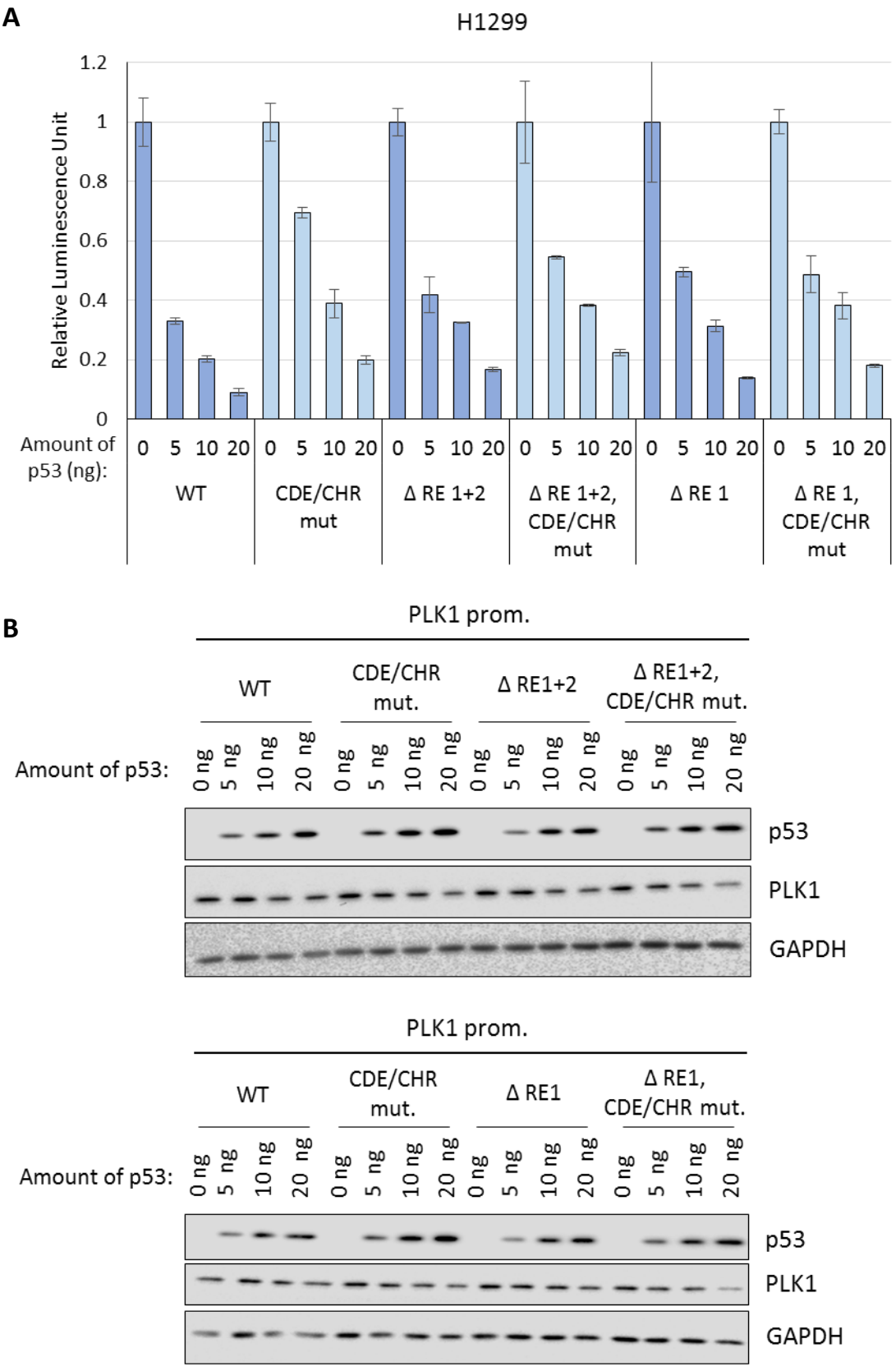


Figure 3-15. p53 dependent repression of *PLK1* occurs even in absence of p53REs and functional CDE/CHR elements.
(A) H1299 cells were transfected with WT and different mutated versions of *PLK1* promoter (as labelled in the figure). Different amounts of p53 expressing plasmid were co-transfected. Cells were lysed, and luciferase assay was performed. **(B)** Western blot analysis of the samples used in the Luciferase assay. These results are representative of 3 independent experiments.

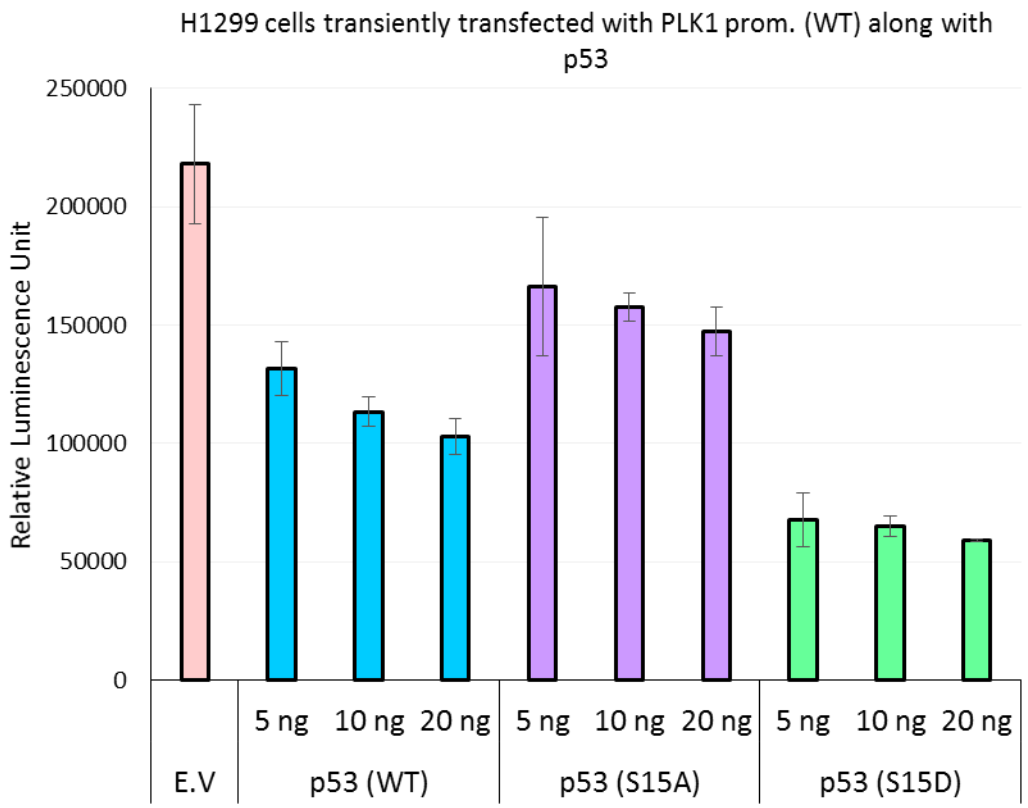
3.3.9 Phosphorylation of p53 at serine 15 is involved in *PLK1* repression

It has been previously shown that phosphorylation of p53 at serine 15 is critical for stimulating the transactivation of genes (Loughery *et al.*, 2014). We were curious to see whether the same is true in p53 mediated repression of *PLK1*.

p53 null H1299 cells were used and transfected with *PLK1* promoter plasmid and increasing levels of plasmids expressing either wild type p53 or p53 that has the serine 15 substituted by alanine (S15A, to prevent phosphorylation) or aspartic acid (S15D, phosphomimic). Empty vector was used as control. Luciferase assay analysis showed that part of the reduction of *PLK1* promoter activity, caused by wild type p53, is lost with S15A mutant. Also, using a phosphomimic mutant (S15D) enhanced the repression of *PLK1* promoter (**Figure 3-16 A**).

Looking closely at the western blot results (**Figure 3-16 B**), p21 levels are higher in S15A mutant compared to wild type p53 which is difficult to explain knowing that serine 15 phosphorylation is required for transactivation of p21 as reported by Loughery *et al.* (2014). Having a blot for p53 (pS15) would have let us to interpret the data better. Also, it would have been nice to have another control (for example a blot for MDM2) or even a luciferase assay for p21 promoter activity with different p53 plasmids (wild type/S15A/S15D).

A



B

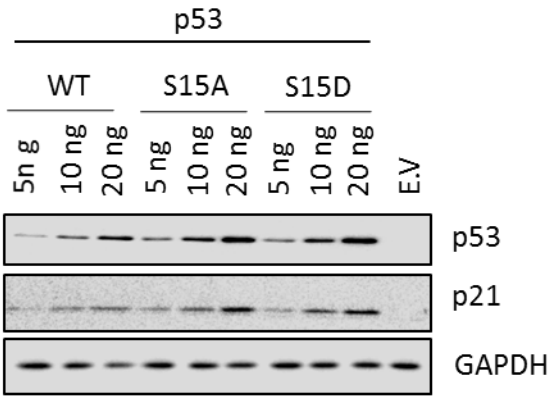


Figure 3-16. Phosphorylated p53 at serine 15 is partly required for p53 mediated *PLK1* repression. **(A)** H1299 cells transfected with plasmid containing wild type *PLK1* promoter in combination with different amounts of plasmids expressing wild type p53, p53 (S15A) or p53 (S15D). Empty vector (E.V) transfected cells were used as control. Luciferase assay was then performed. **(B)** Samples of the luciferase assay were used for western blotting to check the transfection efficiency. These results are representative of 3 independent experiments.

3.3.10 p53 requires an intact N-terminus and DNA binding domain to repress *PLK1*

Since many of the domains of p53 are required for its activity (as explained in **section 1.3.1**), we were interested to see which part of p53 molecule is responsible for the repression of *PLK1*. A number of plasmids were used each containing mutation(s) in p53 gene (**Figure 3-17**) and measured against the performance of wild type p53. One of the plasmids contained two mutations (L22Q/W23S) in transactivation domain 1, TAD1, which binds to the co-activator p300; (these mutations correspond p53 residues 25 and 26 in murine cells and are required for p53 dependent transcription of genes (Hammond et al., 2006). Another plasmid we used had the proline rich domain deleted (∆Pro.). Two other plasmids were used that had mutations in the DNA binding domain and are believed to cause gain of oncogenic functions; R175H causes a conformational change in the site-specific DNA binding domain so makes it unable to bind to the DNA. R273H does not cause a conformational change but eliminates a residue that is important for DNA contact (Muller and Vousden, 2013) (**Figure 3-17**).

p53 null H1299 cells were transfected with *PLK1* promoter along with increasing amounts of wild type or mutated p53 plasmids. Luciferase assay results for plasmids having mutations in N-terminus of p53 (L22Q/W23S and ∆Pro) showed that *PLK1* promoter represses with wild type p53 (more repression in wild type *PLK1* promoter compared to CDE/CHR mutant), whereas none of the mutants of p53 could cause repression of either wild type or CDE/CHR mutated *PLK1* promoter (**Figure 3-18 A, B**). The response of empty vector to different amounts of p53 expressing plasmids was checked as control.

The response of *PLK1* promoter to p53 expressing plasmids that has mutations in DNA binding domain was similar to the N-terminus mutated p53; where wild type p53 could repress the PLK1 promoter, there was no repression seen by any of the DNA binding domain mutated plasmids we used **(Figure 3-19 A,B)**.

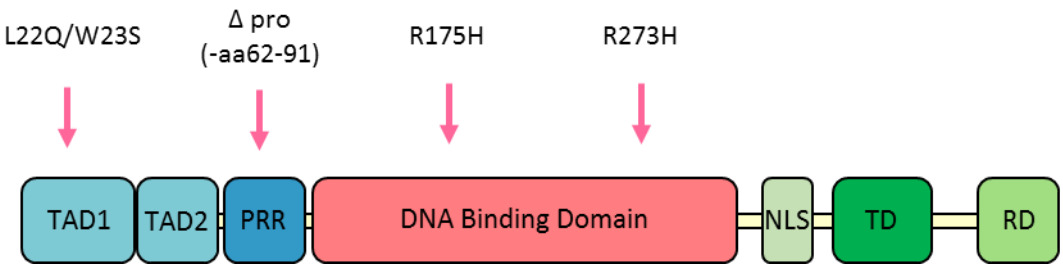
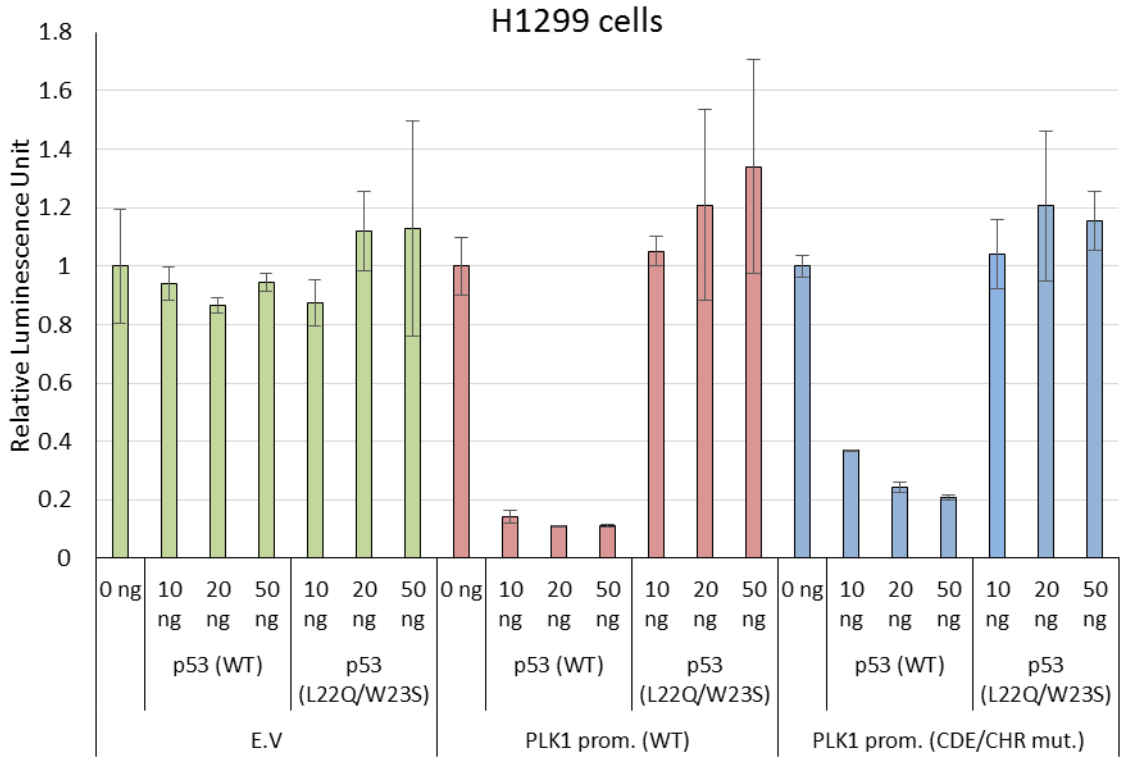
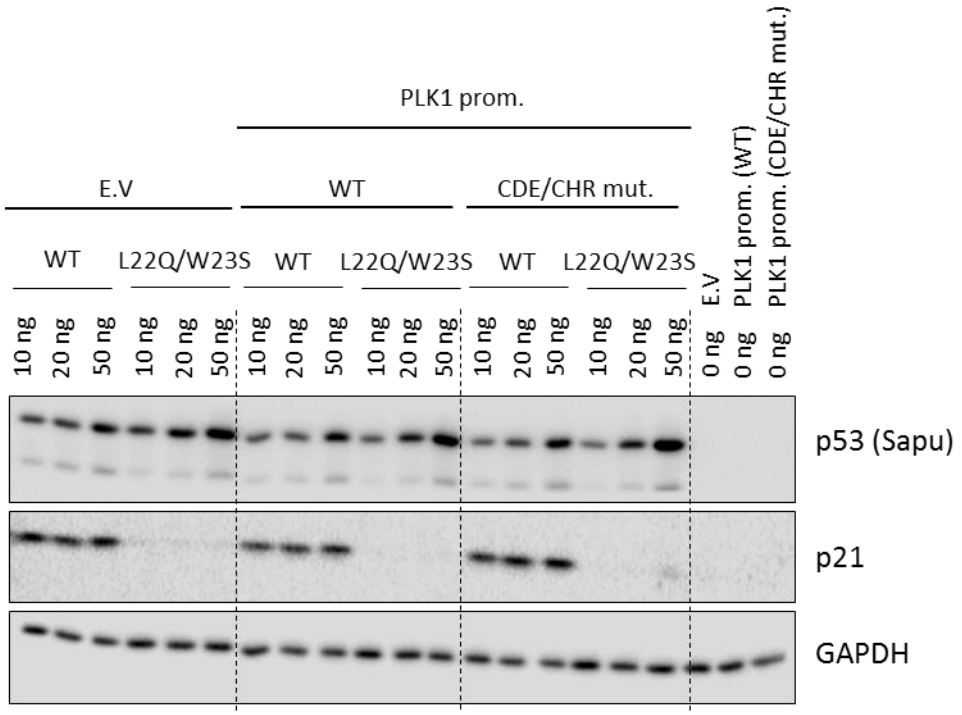


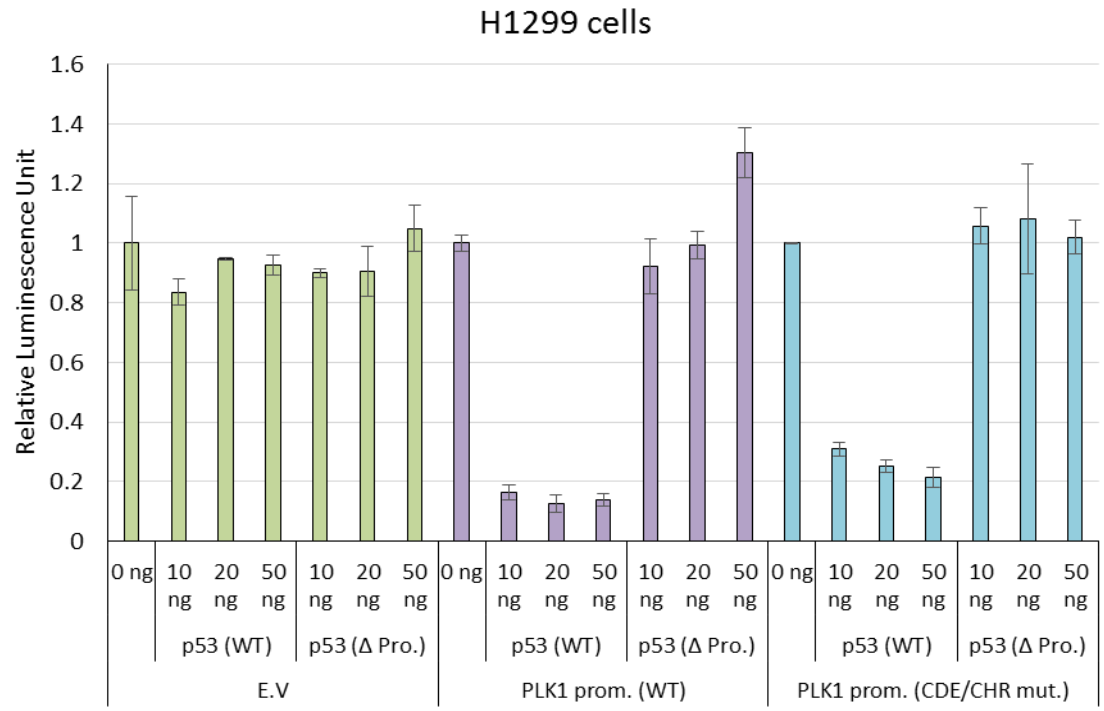
Figure 3-17. Schematic representation of the p53 mutants used.

A


H1299 cells
Relative Luminescence Unit
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 ng
10 ng
20 ng
50 ng
p53 (WT)
p53 (L22Q/W23S)
E.V
PLK1 prom. (WT)
PLK1 prom. (CDE/CHR mut.)


PLK1 prom.
E.V
WT
CDE/CHR mut.
WT
L22Q/W23S
10 ng
20 ng
50 ng
0 ng
E.V
PLK1 prom. (WT)
PLK1 prom. (CDE/CHR mut.)
p53 (Sapu)
p21
GAPDH

B



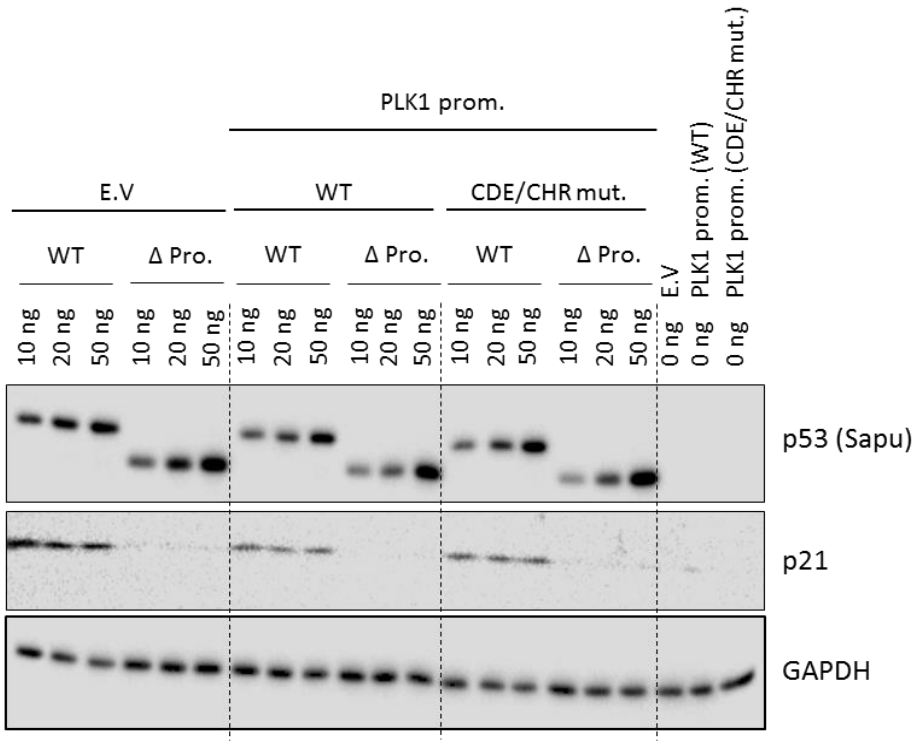


Figure 3-18. Repression of *PLK1* by p53 requires an intact N-terminus of p53.
H1299 cells were transfected with *PLK1* promoter (WT or CDE/CHR mut.) or empty vector as control. Different amounts of WT or mutated (**(A)** L22Q/W23S, **(B)** Δ Pro.) p53 expressing plasmids were transfected as well, empty vector (E.V, 0 ng of p53) was transfected as control. Cells were then lysed and proceeded for Luciferase assay and western blot analysis. Because of the differences in luciferase expressions of each plasmid, it was very difficult to compare if we normalised the values to the 0 ng of E.V. So, the luciferase results have been normalised to the 0 ng of p53 for each E.V, WT or CDE/CHR mut plasmid. These results are representative of at least 2 independent experiments.

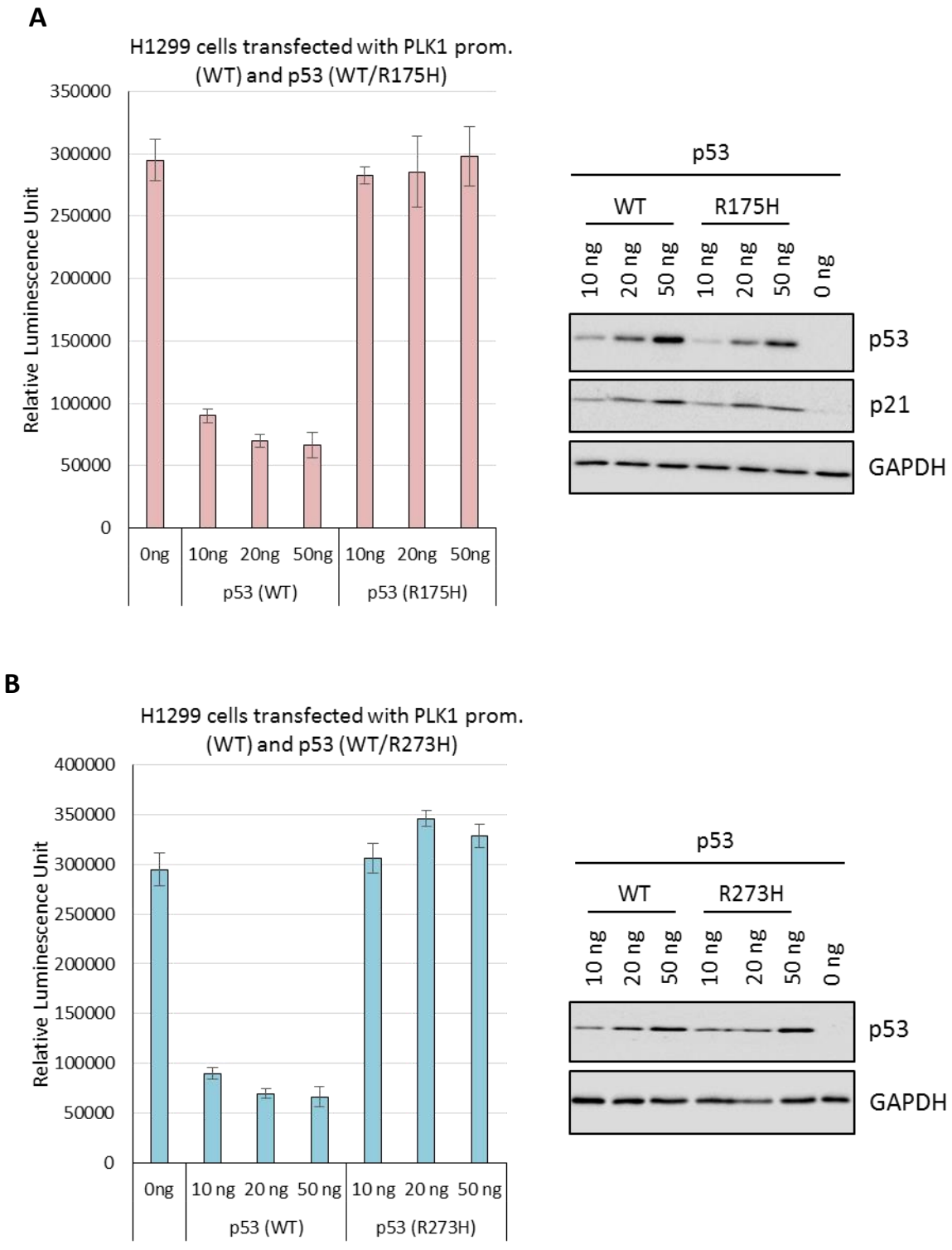


Figure 3-19. Repression of *PLK1* by p53 requires an intact DNA binding domain of p53.
H1299 cells were transfected with PLK1 promoter along with different amounts of WT or mutated (**(A)** R175H, **(B)** R273H) p53 expressing. Empty vector (E.V, 0 ng of p53) was transfected as control. Cells were then lysed and proceeded for Luciferase assay and western blot analysis. These results are representative of at least 2 independent experiments.

3.4 Discussion

In this chapter, we have confirmed that p53 can cause downregulation of PLK1 in response to Nutlin, a pharmacological inhibitor of MDM2. This downregulation is dose dependent and is absent in cells that lack full length p53 or in cells in which p53 expression has been silenced. Surprisingly, DNA damaging agents caused induction of p53 but the effect on PLK1 levels varied between the different agents used. As all of these agents (Nutlin and DNA damaging agents) stabilise p53, and given that p53 clearly represses PLK1 (McKenzie *et al.*, 2010; Fischer, Quaas, Nickel, *et al.*, 2015; Lin *et al.*, 2014), we were very curious to understand why these different agents give rise to apparently different responses. Here using a more quantifiable method would have been helpful (for example using densitometry for western blots) to identify the fold decrease in protein levels.

The results of the cell cycle analysis are particularly interesting as they suggest that different p53 stabilising agents arrest the cells in different phases of the cell cycle. This led us to the hypothesis that the apparent PLK1 downregulation by p53 (as determined by measuring PLK1 levels by western blots) could reflect the PLK1 levels at the point of the cell cycle at which the arrest occurs. Thus if an agent results in an enriched population of cells in a phase of the cell cycle where PLK1 levels are low, we observe an apparently higher downregulation and vice versa (**Figure 3-5**).

This hypothesis was examined by silencing p21, classic downstream target of p53 which is involved in cell cycle arrest particularly in G1 phase. Cell cycle analysis showed that p21 is responsible for the predominantly G1 arrest in the Nutlin treated cells. Knowing that Nutlin treatment on p21 silenced cells reduces

G1 population (a phase of the cell cycle where PLK1 levels are low), western blotting was carried out to check the levels of PLK1 in absence of p21. The data indicate that repression of *PLK1* by p53 is partly p21 dependent and p53 can still repress the *PLK1* promoter in absence of p21, as the *PLK1* repression by p53 is not suppressed completely in Si p21 treated cells**.** However, one might argue that p21 knockdown might not be efficient enough and the small residual p21 levels could be enough to repress PLK1. So, the HCT116 p21-/- cells were used. Also, the role of p21 in p53 mediated repression of the PLK1 promoter was investigated and similar conclusions were drawn (partial involvement of p21). p21 independent repression of *PLK1* by p53 has been reported previously by our laboratory (McKenzie *et al.*, 2010).

These findings raised the question of whether p53 represses the *PLK1* promoter directly or through DREAM interacting with cell cycle dependent elements (CDE/CHR) or through other mechanisms. This made the basis of our investigations regarding p53 mediated *PLK1* repression mechanism. We used a system in which the *PLK1* promoter (wild type or CDE/CHR mutant) cloned in a reporter vector, was used as a readout for *PLK1* promoter activity. Our data showed a cell line dependency and suggested that different cell lines may vary the mechanisms they use to downregulate *PLK1* by p53. The data from the experiments using the HCT116 cells show that *PLK1* repression are largely or even completely dependent on the presence of an intact CDE/CHR element (**Figure 3-9 A**), and therefore agree with the DREAM complex model proposed by Fischer *et al.* (2015). On the other hand, the data from the experiments using the U2OS cells (**Figure 3-9 B**) showed that following mutation of the CDE/CHR elements, there remains a significant level of p53-dependent *PLK1* repression. This repression was not dependent on p53REs either (**Figure 3-14**).

Furthermore, the data from the experiment by which p53 were ectopically expressed in p53 null H1299 cells (**Figure 3-10**), indicate partial involvement of CDE/CHR elements in *PLK1* repression. These data suggest therefore that, while DREAM complex acting via the CDE/CHR elements may provide a level of repression in the U2OS and H1299 cells, there is another mechanism(s) in these cells that represses *PLK1* independently of CDE/CHR.

Overall the mechanism of *PLK1* repression by p53 seems to be complex as the data published in the literature look conflicting and varies between different studies. In the interest of time, our focus in this chapter was mainly on p53-p53REs and p53-p21-DREAM-CDE/CHR mechanisms. Further investigations are required to fully understand the mechanism(s) by which p53 represses *PLK1* and what is responsible for choosing different mechanisms by different cell lines.

Altogether, the conflicting data in the literature regarding the mechanism of *PLK1* repression by p53, could indicate that there might be several pathways involved. Involvement of more than one pathway in p53-mediated repression, has also been reported in other genes, too. Investigations of p53 mediated repression of *CDC25C* using luciferase reporter assay by Clair *et al.* showed that p53 downregulates CDC25C by two different mechanisms. One of the mechanisms is direct binding of p53 to the p53 binding sites. The second mechanism is through CDE/CHR elements and was found to be independent of p21, as p53 could still repress the promoter in the HCT116 p21 null cells. However, p21 alone could repress the expression of the reporter gene, this repression abolished in CDE/CHR mutated promoter. The authors proposed that repression of CDC25C by p53 through CDE/CHR elements could occur via

both p21 dependent and p21 independent mechanisms (Clair *et al.*, 2004). *CDC25C* has also been reported by an independent study to be repressed by direct interaction of p53 to the CCAAT boxes (Imbriano *et al.*, 2005).

Since p53 gets stabilised by post translational modifications, it would be interesting to know which post-translational modifications are involved in the repression of *PLK1* by p53. We have shown that phosphorylation of p53 at serine 15 is involved in *PLK1* downregulation. As it has been published before, serine 15 phosphorylation is necessary for p53 mediated transactivation of genes (Loughery *et al.*, 2014). Hence, Involvement of p53 phosphorylation at serine 15 in *PLK1* repression, fits with a p53 transactivation-based mechanism (as do the results with p53 mutants). However, it does not make it clear whether p53 phosphorylation at serine 15 is required for direct p53 repressive function too, or it is required simply for transactivation of genes that can repress *PLK1* (an indirect regulation). A similar experiment but with CDE/CHR mutated PLK1 promoter would help to eliminate the possibility that introducing each of these p53 expressing plasmids to the cells might result in different cell cycle profile and hence the *PLK1* promoter activity relevant to that particular point of the cell cycle.

Overall the findings of this chapter show that *PLK1* repression by p53 through CDE/CHR elements or by other mechanisms appear to be cell line dependent and there might be more than one pathway involved. However, the complete understanding of this repression requires further investigations.

Chapter 4: PLK1 Inhibition-Induced Mitotic Arrest and DNA Damage Response Differs from Microtubule Poisons

4.1 Background

4.1.1 Cell division cycle and checkpoints

The cell division cycle (also called cell cycle for simplicity) is the process by which all living cells replicate and divide by an orderly sequence of events to produce two identical daughter cells. In eukaryotic cells the cell cycle is divided into two major phases: Interphase and mitotic (M) phase. Interphase takes about 95% of the cell cycle (time wise) and can be subdivided into 3 parts; G1 in which cells undergo growth and normal metabolic roles, S phase is DNA replication phase and G2 is the stage when cells grow and prepare to undergo mitosis (Diaz-Moralli *et al.*, 2013). M phase lasts just about 5% of the cycle (Heijink, Krajewska and Van Vugt, 2013) and can be subdivided into mitosis and cytokinesis which are responsible for nucleic and cytoplasmic divisions respectively.

The cell cycle is controlled by a complex network of regulatory proteins called checkpoints. These are mechanisms that can prevent progression to the next stage of the cell cycle, if impairment have been detected (Kastan and Bartek, 2004). The most prominent checkpoints in eukaryotic cells are located at the boundary of G1 and S phase, at the G2/M transition and between metaphase and anaphase of mitosis. These checkpoints together with associated regulatory proteins such as cyclins and cyclin dependent kinases (CDKs) work toward an error free cell division. Without these control mechanisms, aberrant cell division and genomic instability could occur leading to cancer (Wenzel and Singh, 2018).

4.1.2 Mitosis

Mitosis itself can be subdivided into 5 stages namely prophase, prometaphase, metaphase, anaphase, and telophase. During this phase of the cell cycle, chromosomes undergo dynamic changes in their morphology and movement (Pines and Rieder, 2001).

Mitosis starts when replicated interphase chromosomes become visible in prophase. Kinetochores assemble on the chromosome centromere at the same time. Nuclear envelop breakdown is the hallmark of prophase termination and initiation of prometaphase. In prometaphase, spindle microtubules attach to kinetochores (Cheeseman and Desai, 2008). In order to ensure the fidelity of sister chromatid separation, the mitotic checkpoint/spindle assembly checkpoint (SAC), monitors the progression of mitosis and remains active until metaphase. In case of any defects in kinetochore-spindle microtubule attachments, SAC senses it and prevents the progression to the next stage until all chromosomes attach correctly to the microtubule spindle (Acquaviva and Pines, 2006). By metaphase, all sister chromatid pairs are aligned in the equator of the cell with all kinetochores correctly attached to the microtubule spindles (Cheeseman and Desai, 2008). Upon satisfaction of SAC, Anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase, is activated and targets its substrates for degradation (Cheeseman and Desai, 2008; Musacchio and Salmon, 2007). In anaphase, sister chromatids move away from each other by the force of microtubules. Splitted sister chromatids start decondensing by the onset of telophase. Subsequently the nuclear envelop re-forms to generate the nuclei of daughter cells which is then followed by cytokinesis (Hayashi and Karlseder, 2013).

4.1.3 Importance of mitosis

About 100 million cells in every human body undergo mitosis every minute. Mitosis is the shortest phase of the cell cycle, but it has an important role in the accomplishment of cell division. The chromosomes that have been replicated in S phase, separate in mitosis and genetic information transfers to daughter cells (O'Connor, 2008; Ganem and Pellman, 2012).

Cells going through mitosis undergo lots of stresses; Nuclear envelop break-down, reorganisation of Golgi and endoplasmic reticulum membrane system, chromosome condensation, changes in the shape of actin and microtubules, cessation of vesicle trafficking, disabled transcription and slowed translation are hallmarks of mitosis (Ganem and Pellman, 2012). Because of these dramatic perturbations to the normal architecture of the cells, mitosis is believed to be the most fragile phase in the cell cycle and activation of apoptosis in response to insults is very common during this period (Chan, Koh and Li, 2012).

As mentioned in the last section, in cells undergoing mitosis the SAC is active until all kinetochores attach properly to the spindle microtubules. Any insult that prevents such correct attachment results in prolonged maintenance of the SAC and causes cells to arrest in mitosis until the damage is repaired and all kinetochores attach correctly, or else cells might undergo mitotic cell death (Musacchio and Salmon, 2007). This is one of the anti-proliferative mechanisms induced by chemotherapeutic agents (Chan, Koh and Li, 2012)

4.1.4 Mitotic arrest and DNA damage:

As discussed, Mitotic cells experience lots of stresses explaining why mitosis needs to be a short period. Prolonging the period of mitosis leads to a stress response in cells and could cause chromosome breaks/DNA damage (Orth *et al.*, 2012; Dalton *et al.*, 2007; Ganem and Pellman, 2012) which can ultimately lead to inhibition of cell proliferation or induction of apoptosis. These outcomes, in fact, can block the proliferation of cells with aberrant chromosomes and can inhibit genomic instability. This mechanism is important for the effectiveness of the anti-mitotic drugs against cancer cells (Hain *et al.*, 2016).

The reasons for DNA damage induced after prolonged mitosis are still under investigations but Orth *et al.* demonstrated that it could be because of outer mitochondrial membrane permeabilisation after the long mitotic arrest which leads to cytochrome c leakage. They concluded that the DNA damage caused by prolonged mitotic arrest is caspase dependent as the damage was inhibited when caspase inhibitors were used (Orth *et al.*, 2012). Hayashi and colleagues used the method of γ-H2AX staining as a marker to detect DNA damage and telomere fluorescence in situ hybridisation (FISH) technique to detect the telomeres (sequence of repetitive nucleotides at the end of the chromosomes). The co-localisation of γ-H2AX foci and telomeres, which indicates the telomeric damage, was found in cells undergoing prolonged mitosis (Hayashi *et al.*, 2012).

Eukaryotic cells have evolved a complex of proteins located at the end of telomeres, termed shelterin. The shelterin complex is composed of double stranded repetitive sequence of 5’-TTAGGG-3’ (with the complementary DNA strand being 3’-AATCCC-5’) followed by a single stranded G-rich 3’ overhang

which is associated with the complex of 6 proteins. Shelterin functions to cap and protect chromosome ends, and enables the cells to differentiate between chromosome ends and DNA breaks (de Lange, 2009*;* Palm and de Lange, 2008).

The 6 subunits of shelterin complex are TRF1, TRF2, POT1, TIN2, Rap1 and TPP1. Among the subunits, TRF2 and POT1 have been reported to be more predominantly involved in chromosome end protection by repressing the ATM and ATR DNA damage signalling pathways and consequent checkpoint activation (Denchi and de Lange, 2007). It has been suggested that during mitotic arrest TRF2 is partially removed from telomeres, causing partial telomere deprotection/dysfunction of telomeres and subsequent DNA damage response at the end of chromosomes (Hayashi *et al.*, 2012).

4.1.5 Targeting mitosis for cancer therapy:

One of the clinically effective approaches which is widely used in cancer therapy is to disrupt mitotic progression (Driscoll *et al.*, 2014). These agents interfere with normal mitotic progression but are ineffective against non-dividing cells (Chan, Koh and Li, 2012). Microtubule poisons such as nocodazole and colchicine are among the first studied agents causing mitotic arrest and consequent DNA damage. These agents bind β-tubulin and act on the polymerisation dynamics of microtubule spindles which is crucial for their proper function (Mukhtar *et al*., 2014). Therefore, they maintain the activity of the SAC by preventing kinetochore attachment. The resulting prolonged mitotic arrest that occurs ultimately causes cells to undergo cell death or slip out of mitosis without completion of anaphase or cytokinesis (Ganem and Pellman, 2012).

Although microtubule poisons are very effective and widely used in cancer therapy, the side effects associated with them are very common and severe. They are meant to selectively affect the cells in mitosis only, but the possibility that they affect interphase cells too, is very high as microtubules are widespread during the whole process of the cell cycle. Therefore, they can have unwanted effects on non-proliferating cells. On the other hand, complications related to resistance to these agents is not uncommon (mainly due to drug efflux pumps or mutations in tubulin). So the attention has been towards finding novel drugs that still target the mitotic progression but with a different mechanism of action compared to microtubule poisons, of such agents are kinase inhibitors (Chan, Koh and Li, 2012; Matson and Stukenberg, 2011).

The evolutionary conserved regulatory function of cyclin dependent kinases (CDKs) opened new avenues to understand the mitotic progression and stimulated a search for other kinases which work together with CDKs to regulate cell division. PLKs are one of the most prominent families of these kinase proteins that as highlighted in **Figure 1-2** have critical functions throughout mitosis (Barr, Silljé and Nigg, 2004).

Scientists studying mitotic arrest, most commonly use microtubule poisons to study the consequences of prolonged mitotic arrest. We were interested in focusing on PLK1 and how inhibitors of PLK1 affect mitosis, and what outcome such inhibition might bring about especially in terms of DNA damage. Immuno-fluorescence studies were carried out in most of the experiments in this chapter to visualize γ-H2AX foci, which we used as a marker for DNA damage.

4.2 Aims

The Aim of this chapter is to determine whether inhibition of PLK1 induces a DNA damage response in mitosis similar to that induced by microtubule poisons and how the mechanisms could be different.

4.3 Results

4.3.1 Inhibition of PLK1 causes the formation of mono-polar spindles

As discussed before (see section 1.2.2), PLK1 has important roles in maturation and separation of centrosomes and assembly of bipolar spindles mainly by recruiting γ-tubulin. Inhibiting PLK1 is expected to disturb the assembly of bipolar spindles as it interferes with the separation of centrosomes. This concept was investigated by treating the cells with the PLK1 small molecule inhibitor, BI2536. A549 and U2OS cells were treated with BI2536 for 2 hours, after which mitotic wash off was carried out. Collected mitotic cells re-pated in the media containing BI2536 for further 2 (A549) or 4 (U2OS) hours. Cells were then fixed and stained with α-tubulin antibody and immuno-fluorescence was carried out. In a similar approach, cells were treated with microtubule de-polymeriser, nocodazole and immuno-stained with α-tubulin antibody. Normal mitotic cells were used as control. **Figure 4-1** shows a representation of the images taken. In normal mitosis we see proper bipolar spindle formation where the cells will divide normally. In the nocodazole treatment, microtubules had not polymerized so the microtubules cannot attach to kinetochores and in BI2536 treated cells we see the formation of monopolar spindles.

Interestingly, looking at the nocodazole treatment in A549 (**Figure 4-1 A)** and U2OS cells **(Figure 4-1 B)**, there seems to be a different morphology of microtubules between the 2 cell lines. In other words, it appears that A549 cells are more sensitive to microtubule de-polymerisation effect of nocodazole as compared to U2OS cells.

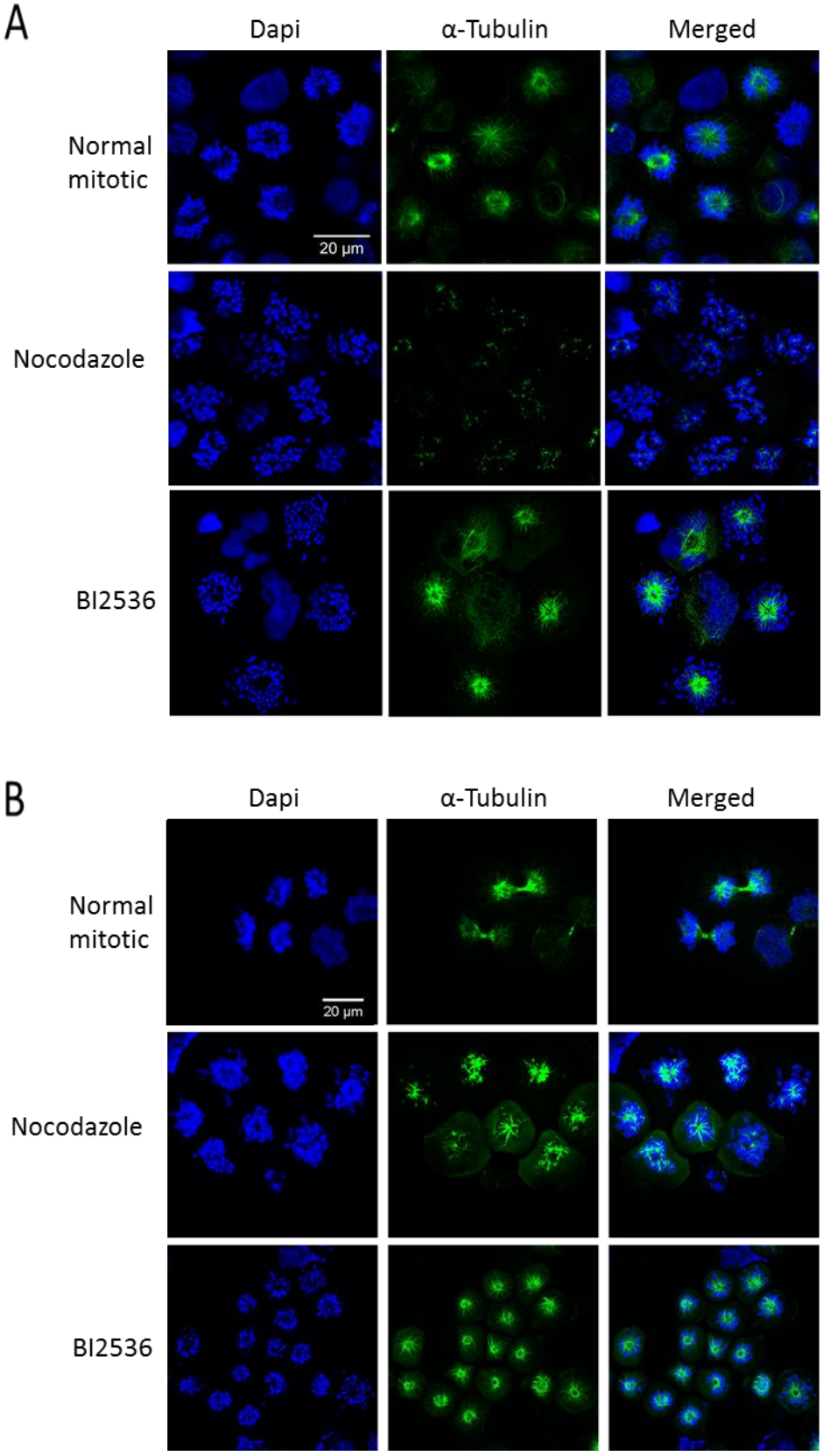


Figure 4-1. Morphology of microtubules of the cells treated with different mitotic poisons.
(A) A549 **(B)** U2OS cells were treated with nocodazole/BI2536 for 2 hours, mitotic wash off was then carried out. Collected mitotic cells were left for another 2/4 hours (A549/U2OS respectively) in media containing drug before cells undergo immuno-staining. Normal mitotic cells were used as control. Representive images are shown.

4.3.2 Inhibition of PLK1 arrests the cells in mitosis

As mentioned in the introduction chapter, in prometaphase and metaphase PLK1 localizes to the kinetochores and spindle poles to regulate assembly of the kinetochores and contributes to the spindle assembly checkpoint (Degenhardt and Lampkin, 2010). When PLK1 is not present, bipolar spindles do not form and thus cells arrest in prometaphase as a result of checkpoint activation (Barr, Silljé and Nigg, 2004). We therefore decided to use a range of concentrations of the PLK1 inhibitor BI 2536 to see the response of the cells to different concentrations at different time points in terms of mitotic arrest. Initially U2OS cells were treated with BI2536 for 2 hours. Then the floating, mitotic cells were collected and re-plated into BI2536 containing media for further 2 or 4 hours (P4M and P6M respectively).

Washing off mitotic cells and re-plating them for further 2 or 4 hours in BI2536 containing media enabled us to see if cells would still be in mitosis after that length of time (2/4 hours). Because if PLK1 does not arrest the cells in mitosis, after 2/4 hours, cells should have exited mitosis. Cells were then collected, cytospun and stained with an antibody against histone H3 phosphorylated at serine 10, a commonly used marker of mitotic cells. Confocal microscopy was then used to analyse the stained cells. Analysis of the images showed that BI2536 causes mitotic arrest even in the lowest concentration used (10 nM) **(Figure 4-2)**.

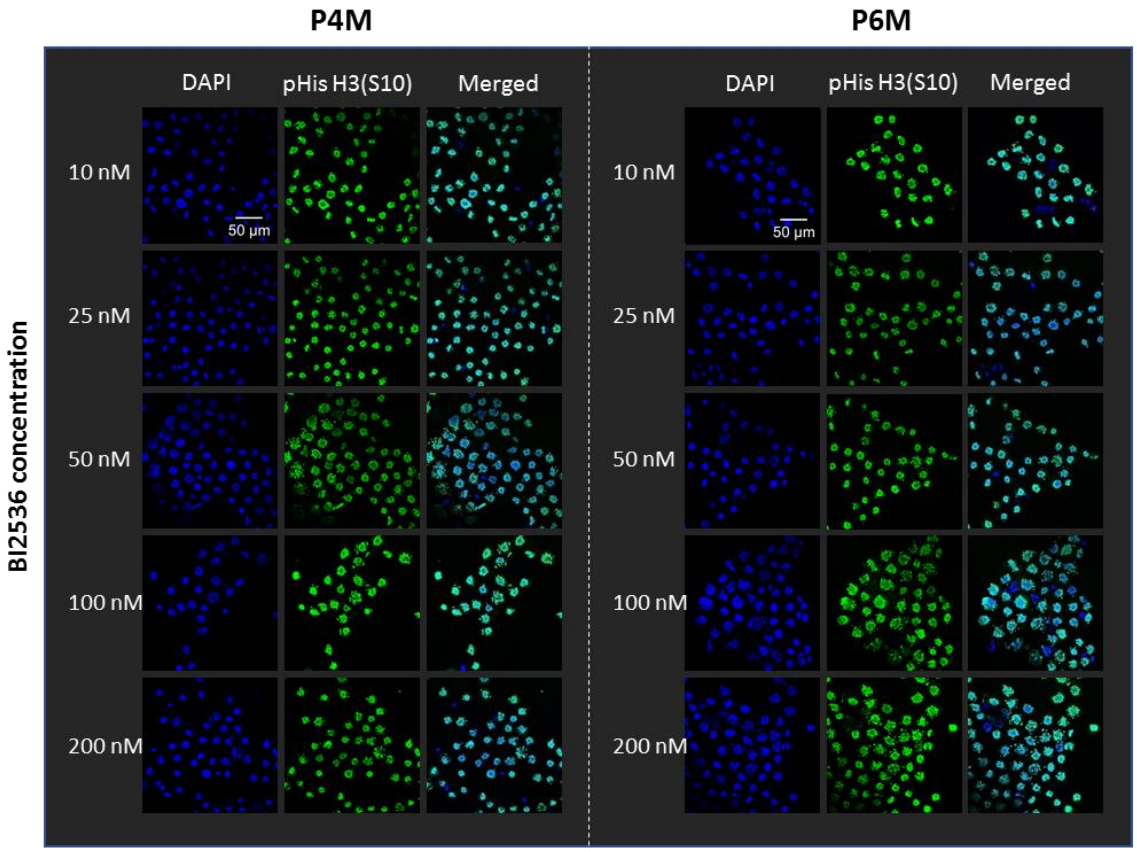


Figure 4-2. PLK1 inhibition results in mitotic arrest.
U2OS cells were treated with different concentrations of BI2536 for 2 hours followed by mitotic wash off and re-plating the mitotic cells in the same media containing BI2536 for another 2 or 4 hours (P4M/P6M). Cells were then collected, cytospun and stained with anti-phospho Ser10-histone H3 antibody and DAPI to visualise the DNA. These results are representative of 2 independent experiments.

4.3.3 BI 2536 causes a time and dose dependent DNA damage

Prolonging the period of mitosis, by using microtubule poisons, has been documented to induces DNA damage (Colin et al., 2015; Dalton et al. 2007). We were interested to check whether this was also the case when cells were arrested in mitosis using the PLK1 inhibitor. Cells were treated with different concentrations of BI2536 and after 2 hours floating mitotic cells were collected and re-plated in BI2536 containing media for further 2 or 4 hours. Normal mitotic cells and nocodazole-treated cells were used as controls. Immuno-staining performed using an antibody against γ-H2AX (pS139), a marker of DNA damage. Analysis of the results, by counting γ-H2AX foci in mitotic cells, showed that PLK1 inhibition causes a dose-dependent DNA damage response (DDR), which further increases over time **(Figure 4-3)**. In other words, the intensity of the DNA damage signalling observed after treating the cells with PLK1 inhibitor is under the influence of both concentration of BI2536 and the duration of mitotic arrest.

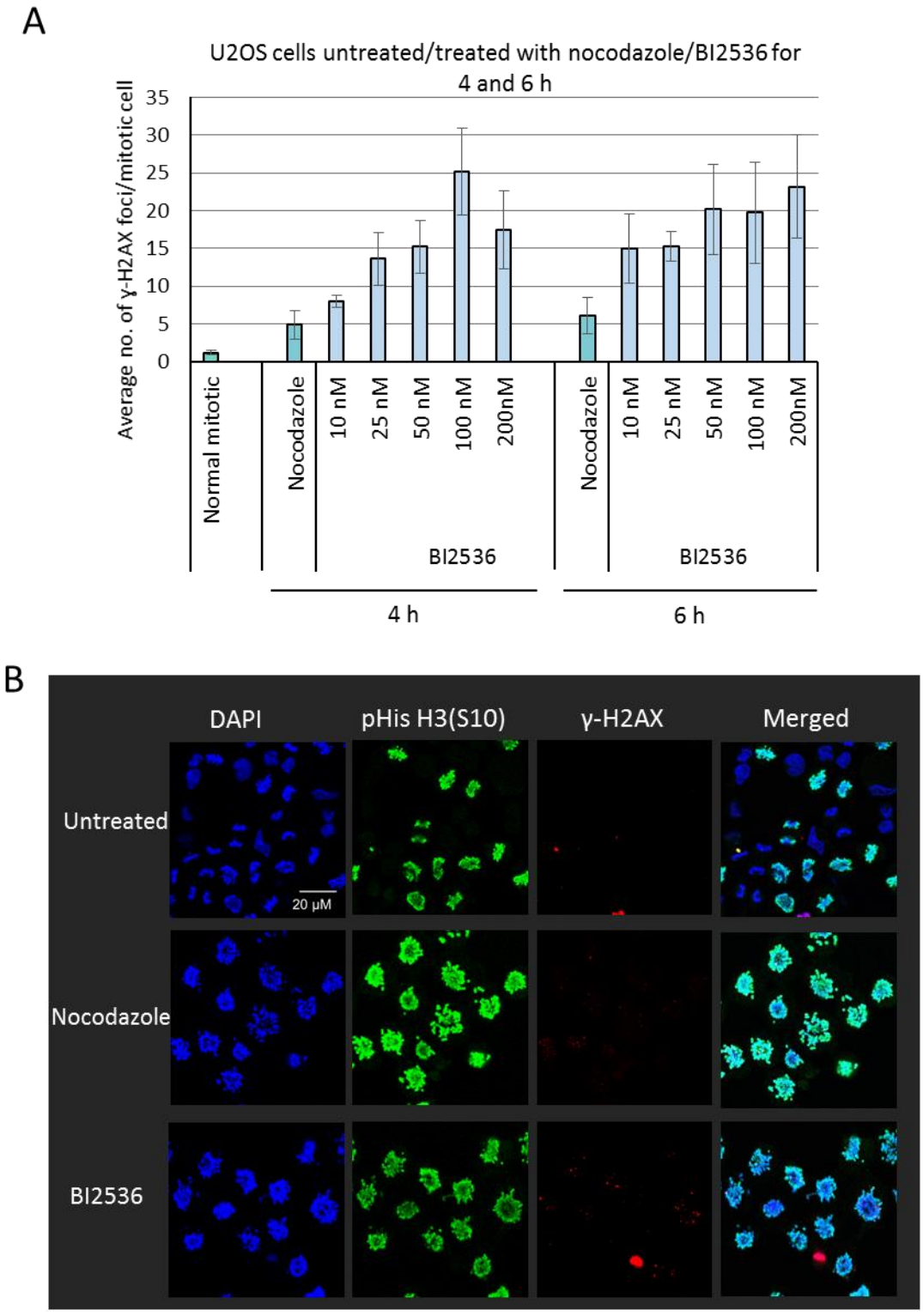


Figure 4-3. Time and dose dependency of BI2536-induced DNA damage response.
(A) U2OS cells were treated with 100ng/ml of nocodazole or different concentrations of BI2536 for 2 hours followed by mitotic wash off and re-plating the mitotic cells in the same media containing BI2536 for another 2 or 4 hours. Normal mitotic cells (Untreated mitotic cells) were used as control. Cells were then collected, cytospun and immuno-stained using anti-phospho-Ser10 histone H3 and anti-γH2AX antibody. **(B)** Representative images of normal mitotic cells, nocodazole/BI2536 treated cells for 2 hours followed by mitotic wash off and incubation for another 4 hours in the same media containing nocodazole or BI2536. These results are average/representative (A/B respectively) of 3 independent experiments.

4.3.4 Inhibition of PLK1 results in both telomeric and non-telomeric damage and partial dissociation of TRF2 from telomers

Cells have evolved a sequence of repetitive nucleotides at the end of the chromosomes called telomeres. These structures function to prevent the chromosome ends being recognised as sites of DNA damage and thereby prevent activation of checkpoint and cellular DNA damage response which could lead to cell cycle arrest and DNA repair activation that may cause disastrous consequences for genome integrity (de Lange, 2009).

Previous work from our laboratory has shown that most of the DNA damage caused by microtubule poisons is localised at the telomeres (Hain *et al.*, 2016). We were interested to know whether this is also the case when cells are treated with the PLK1 inhibitor. To test this, we stained the treated cells with an antibody against γ-H2AX to detect DNA damage followed by Fluorescence In Situ Hybridisation (FISH) with a telomere specific probe to visualise telomeres. Co-localisation of γ-H2AX foci and telomeres indicates telomeric DNA damage, the so-called telomere dysfunction induced foci (TIFs). In contrast to the DNA damage caused by microtubule poisons, which is mostly telomeric, there seems to be no obvious difference between the number of telomeric and non-telomeric DNA breaks in PLK1 inhibitor treated cells (**Figure 4-4 A, B**).

As mentioned in section 4.1.4, eukaryotic cells have evolved a complex of proteins at the end of their telomeres, called shelterin, that functions to cap and protect chromosome ends, and enables the cells to differentiate between chromosome ends and DNA breaks (de Lange, 2009*;* Palm and de Lange, 2008). Extensive works from our laboratory and another laboratory suggested that during mitotic arrest TRF2 (a subunit of shelterin) is partially removed from

telomeres causing partial telomere deprotection and subsequent DNA damage (Hayashi *et al.*, 2012; Hain *et al.*, 2016). Since the proportion of telomeric to non-telomeric DNA damage induced by nocodazole and BI2536 is different, the curious question was then whether PLK1 inhibitors behave the same as microtubule poisons in causing to displace TRF2 from the telomeres? To investigate this, mitotic cells from nocodazole or BI2536 treated cells were stained for TRF2 and number of TRF2 foci in each mitotic cell was counted. Cells treated with nocodazole and BI2536, both caused loss of TRF2 from telomeres, with nocodazole causing more TRF2 loss compared to BI2536 (**Figure 4-5**). There are no error bars on the bar graph since the experiment has been done once only. Drawing any conclusion from this experiment requires further experimentation.

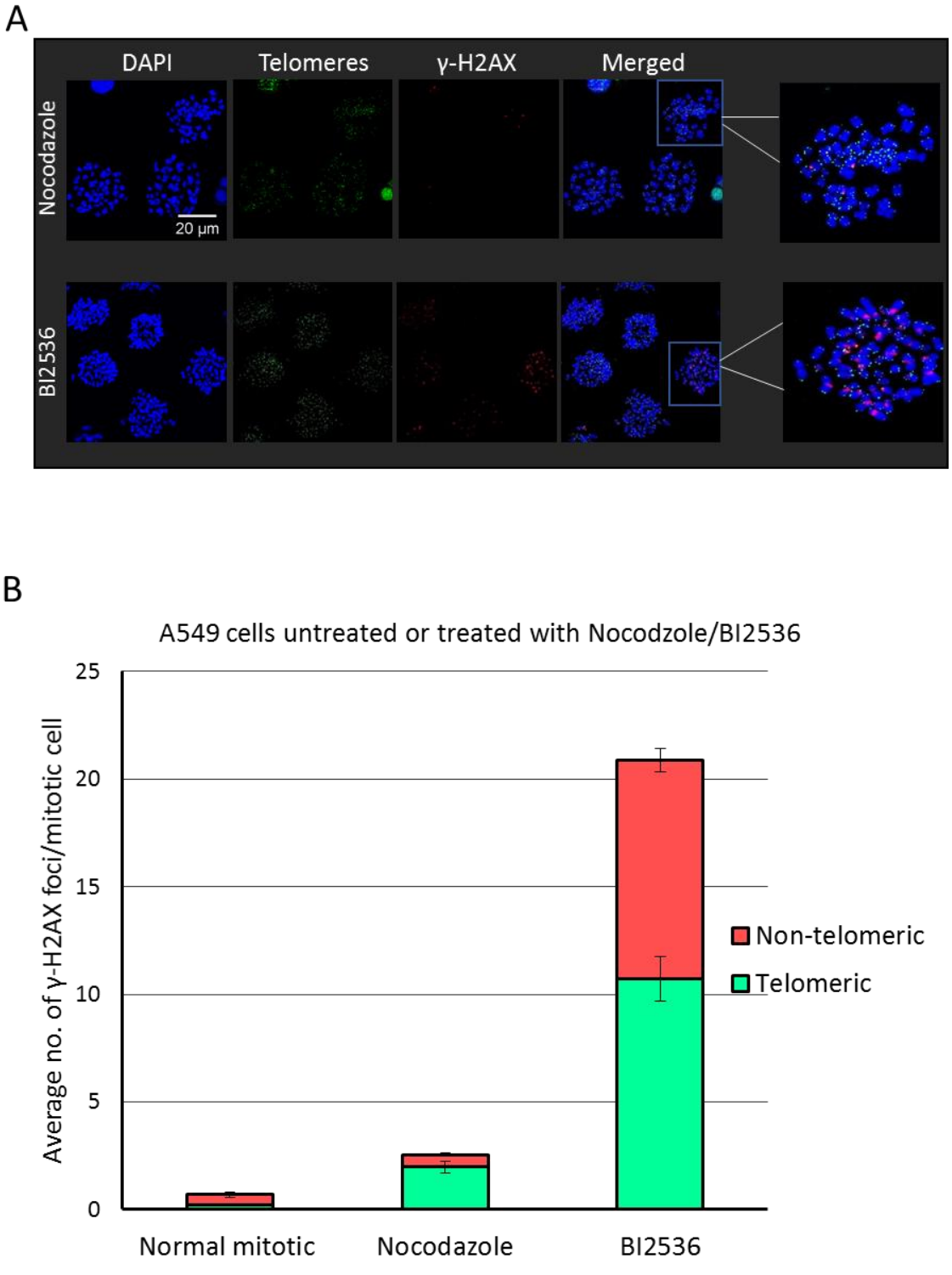


Figure 4-4. DNA damage induced by PLK1 inhibition is both telomeric and non-telomeric.
A549 cells were treated with 10 nM BI2536 or 100 ng/ml nocodazole for 2 hours. Mitotic wash off carried out and mitotic cells replated in media containing drug for further 2 hours. Samples were collected, cytospun and immuno-stained for γ-H2AX. FISH was performed using a telomere probe (5'-TTAGGG-3', green) to detect telomeres. Co-localization of γ-H2AX and FISH shows telomere dysfunction induced foci (TIFs). **(A)** Representative microscopic images are shown. (**B)** Quantification of TIF versus non-TIF. These results are representative/average (A/B respectively) of 3 independent experiments.

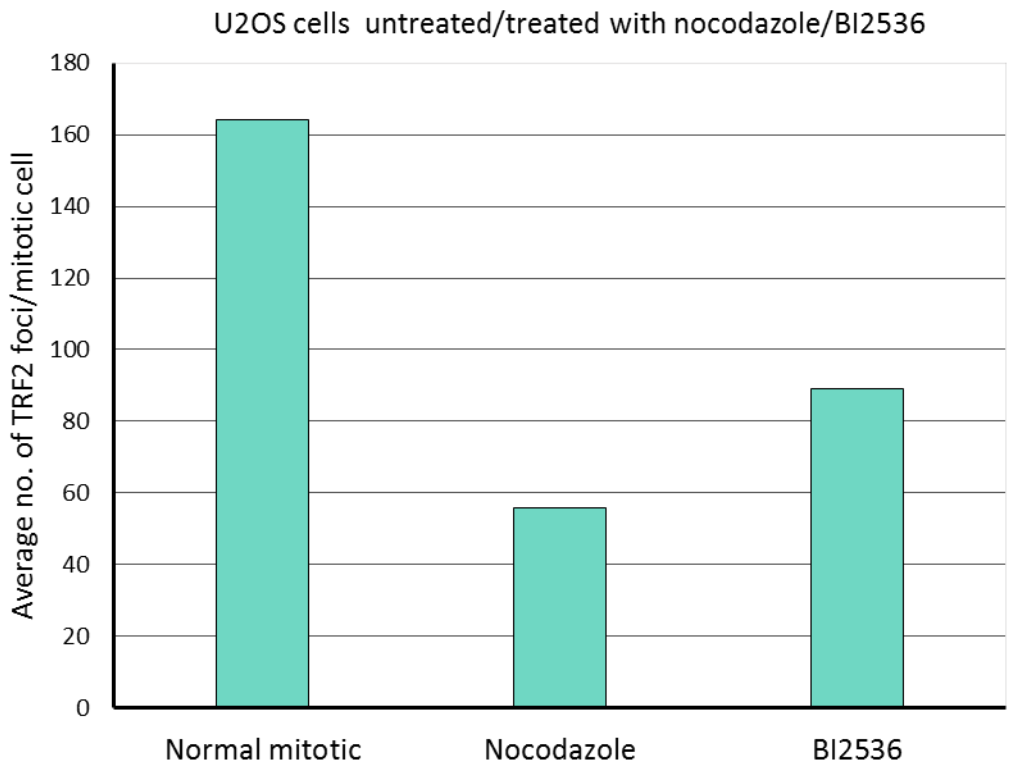


Figure 4-5. Mitotic arrest induced by PLK1 inhibitor results in loss of TRF2.
U2OS cells were treated with 100 ng/ml Nocodazole or 25 nM BI2536 for 2 hours. Mitotic cells were then separated and re-plated in the media containing drugs for further 4 hours. Cells were then collected, cytospun and immuno-stained with antibody against TRF2. Number of TRF2 foci was then counted in at least 25 mitotic cells. The data shown is for 1 experiment (minimum of 25 cells counted).

4.3.5 DNA damage caused by PLK1 inhibition is not caspase dependent

The DNA damage-induced mitotic arrest by microtubule poisons has been reported to be caspase dependent (Colin *et al*., 2015; Hain *et al.,* 2016; Orth *et al.,* 2012). We were therefore interested to see if caspases play any role in the DNA damage caused by PLK1 inhibition-induced mitotic arrest. To test this, we used 2 different approaches. One approach was to use the pan-caspase inhibitor z-VAD-fmk to check the caspase dependency. Another approach was to use cells that express a non-cleavable mutant of ICAD (inhibitor of CAD) to check the CAD dependency of the damage induced (CAD works downstream of caspases and will be explained below).

Firstly, cell were treated with mitotic poisons, nocodazole and BI2536, in presence or absence of z-Vad fmk. Surprisingly we noticed that the DNA damage induced by BI2536 is not inhibited by z-VAD-fmk in contrast to the nocodazole control (**Figure 4-6**). These data indicate that inhibition of PLK1 induces a different, caspase independent DNA damage pathway in mitotically arrested cells (as compared with nocodazole).

To further investigate caspase dependency of the damage and confirm our data, involvement of CAD (caspase activated DNase) was investigated by using a cell line which expresses YFP-ICAD. ICAD (inhibitor of CAD) acts as the endonuclease CAD inhibitor. In response to apoptotic stimuli, caspases cleave ICAD, as a result CAD is not inhibited anymore and can cause DNA fragmentation and apoptosis (**Figure 4-7**). The cells that we used were expressing a non-cleavable mutant of ICAD (D117E/D224E) that inhibits CAD endonuclease independently of caspase activity (kindly provided by team members; Dr. Karolina Hain and Dr. Desiree Rutschow). They have been stably

transfected with a doxycycline-inducible YFP-ICAD mutant integrated at FRT sites in U2OS Flp–In cells. As in these cells ICAD cannot be cleaved by caspases, so CAD remains inhibited and we expect to see reduction of DNA damage if the damage is CAD dependent.

As indicated in **Figure 4-8**, ICAD mutant cells show a significant reduction of γ-H2AX foci after treatment with nocodazole compared to control (YFP cells). This is consistent with previous results in our laboratory (Hain *et al.*, 2016). However, we do not see a significant reduction in γ-H2AX foci in BI2536 treated ICAD-mutant cells. This finding agrees with the lack of effect of caspase inhibitor on γ-H2AX foci after treatment with BI2536 and suggests that PLK1 inhibition induces mitotic DNA damage in a caspase-independent manner.

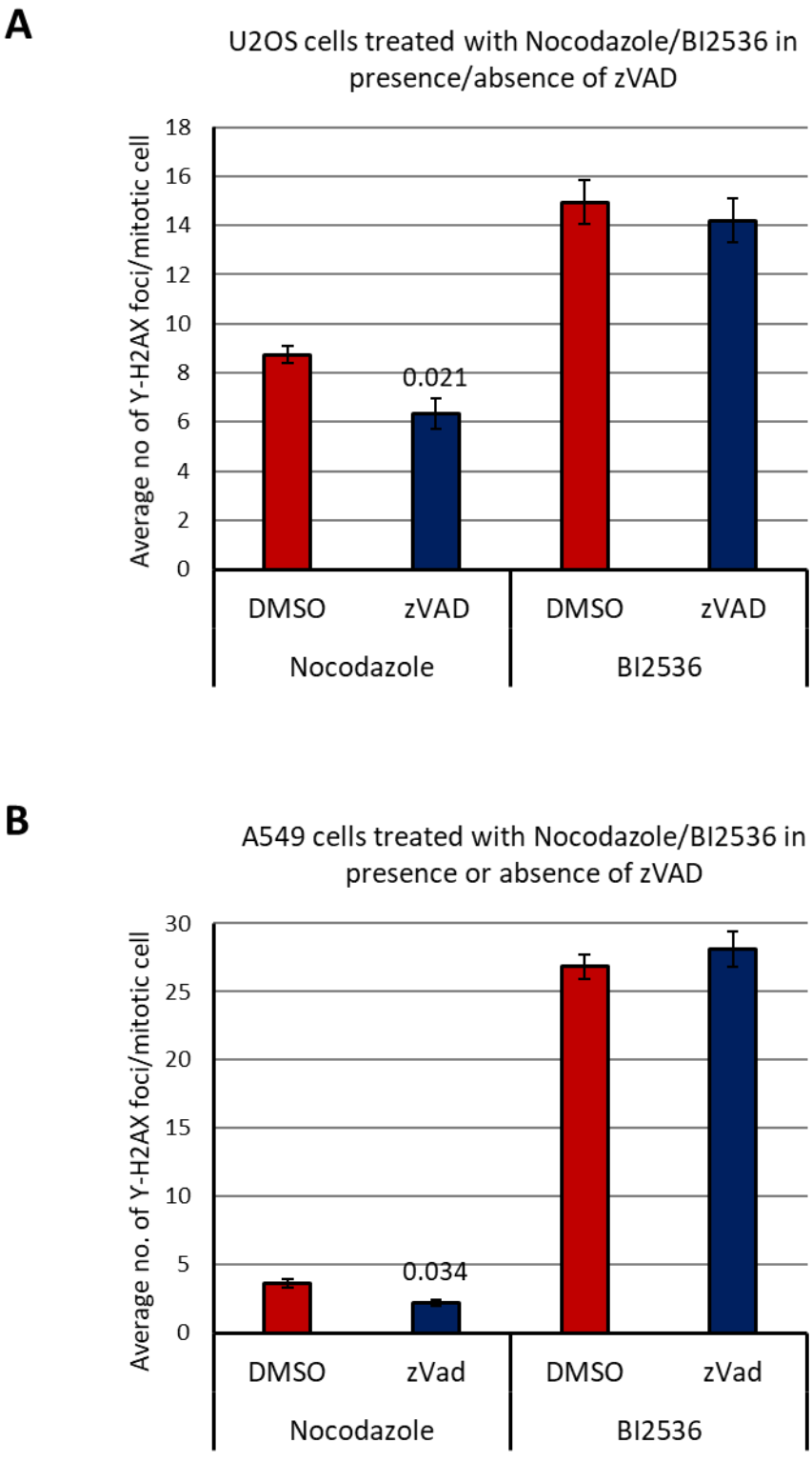


Figure 4-6. DNA damage induced by BI2536 is not caspase dependent.
(A) U2OS cells were treated with 25 nM BI2536 or 100 ng/ml nocodazole (in presence or absence of 20 µM zVAD-fmk) for 2 hours. Mitotic wash off was then carried out and mitotic cells were re-plated in the same media (containing drugs) for further 4 hours. Cells were then collected and proceeded for immuno-fluorescence. **(B)** A549 cells were treated with 10 nM BI2536 or 100 ng/ml nocodazole for 2 hours, in presence or absence of 20 µM zVAD-fmk. Mitotic wash off was then carried out and mitotic cells were re-plated in the same media (containing drugs) for further 2 hours. Cells were then collected and proceeded for immuno-fluorescence. These results are average of 3 independent experiments. The numbers on the bars refer to p-value as determined by t-test.

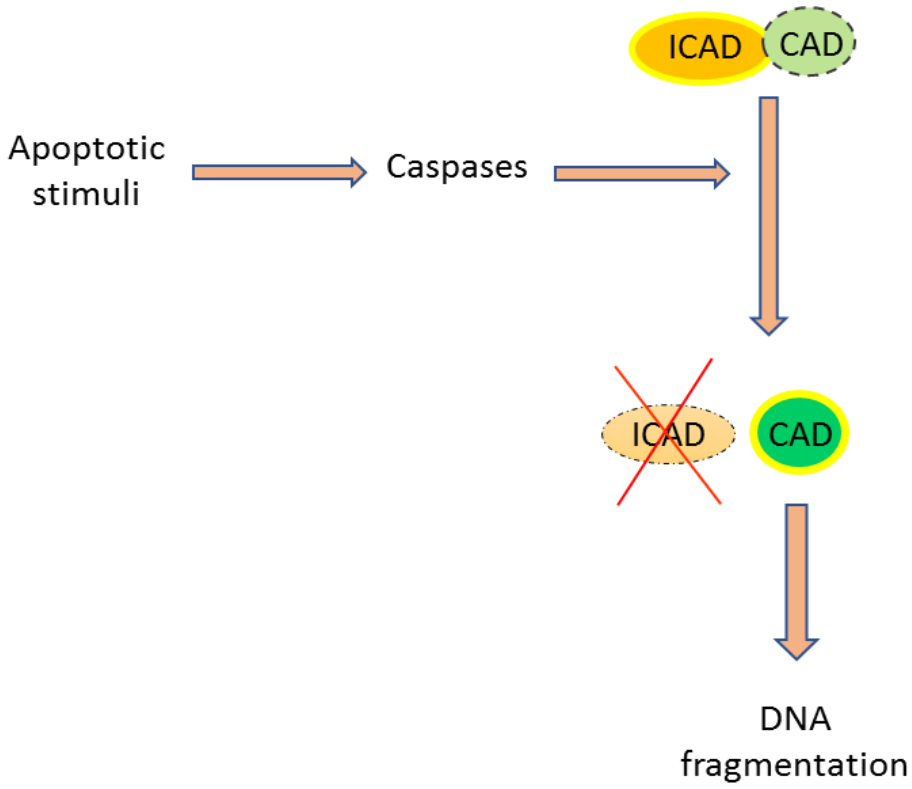
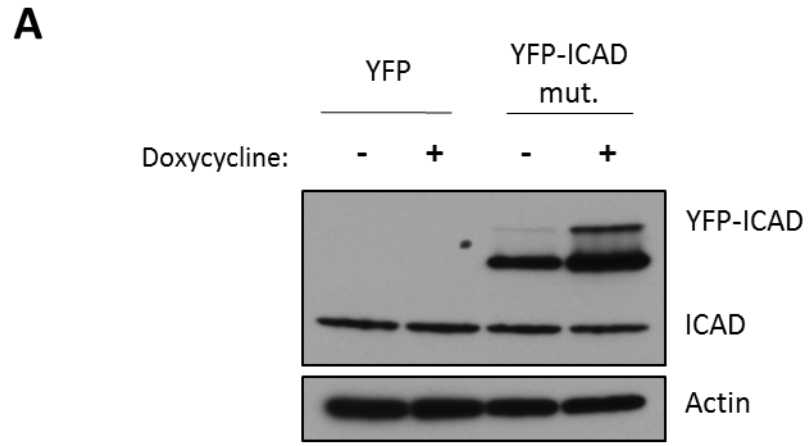


Figure 4-7. Schematic representation of caspase dependent DNA damage.
In response to apoptotic stimuli, caspases mediate the cleavage of ICAD, an inhibitor of endonuclease CAD. As the result, activated CAD can result in DNA fragmentation and apoptosis.



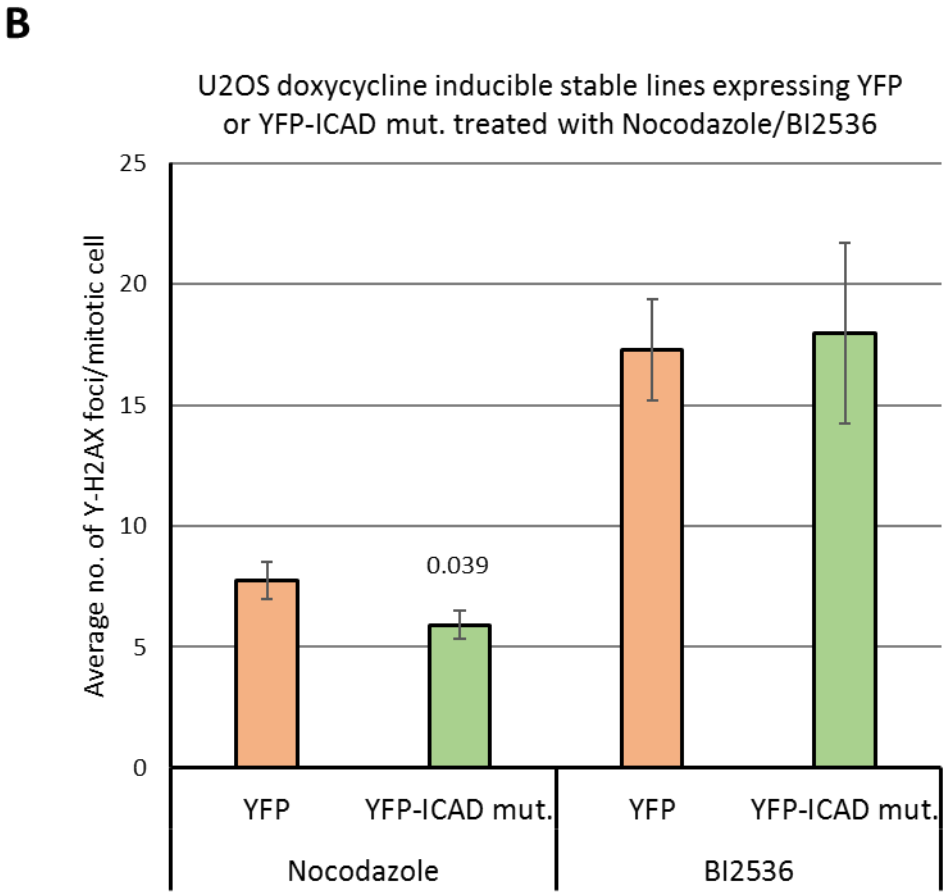


Figure 4-8. DNA damage induced by BI2536 is not CAD dependent.
(A) Asynchronous stable U2OS YFP and YFP-ICAD mutant cells were collected and treated with or without doxycycline. Immuno-blotting was performed to check ICAD expression in ICAD mutant cells. **(B)** Cells were treated with 25 nM BI2536 or 100 ng/ml nocodazole for 2 hours. After mitotic wash off, mitotic cells were re-plated in the media containing BI2536 or nocodazole respectively for further 4 hours. Cells were then collected, cytospun and immuno-stained with anti-γ-H2AX antibody. These results are average of 4 independent experiments (western blotting has been performed once). The number on the bar refers to p-value as determined by t-test.

4.3.6 ATR and DNA-PK are involved in DNA damage response caused by PLK1 inhibition

Several protein kinases are believed to sense DNA damage among which ATM (ataxia telangiectasia, mutated), ATR (ATM- and Rad3-related) and DNA double-strand break repair enzyme DNA-PK (DNA dependent protein kinase) are well known. These kinases belong to the phosphatidylinositol-3-kinase-related protein kinases (PIKKs) family which phosphorylate H2AX at ser139 (γ-H2AX) during DNA damage response (Yang *et al.*, 2003). We were interested in knowing whether the mitotic DNA damage induced by PLK1 inhibition is under the influence of these kinases.

Chemical inhibitors of ATM, ATR and DNA-PK were used to test whether inhibiting these kinases have any effect on the number of γ-H2AX foci formed by inhibition of PLK1. **Figure 4-9** indicates that following the inhibition of DNA-PK and ATR, the number of γ-H2AX foci in BI2536 treated cells reduces whereas ATM inhibition showed no differences. Nocodazole induced γ-H2AX foci on the other hand was found to be influenced by DNA-PK inhibitor only.

Since the results are marginal, having a control experiment would have been helpful to determine the effectiveness of the kinase inhibitors. For example, western blot analysis could have been performed for the kinases used, to check the level of inhibition by their respective inhibitors.

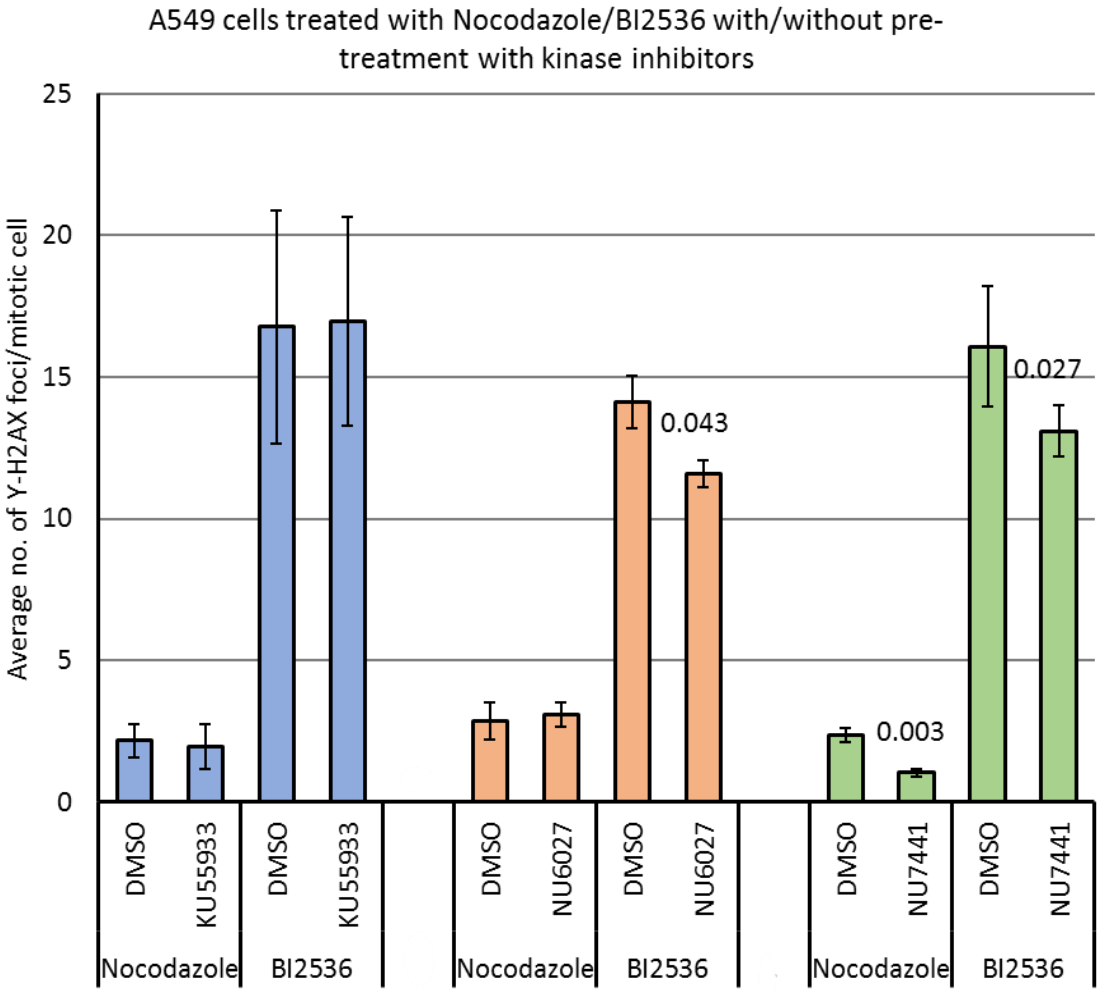


Figure 4-9. DNA damage response caused by PLK1 inhibition is partly dependent on ATR and DNA-PK. A549 cells were treated with 10 nM BI2536 or 100 ng/ml nocodazole with or without 30 minutes pre-treatment with different DNA damage sensing kinase inhibitors; ATM inhibitor KU55933 (10 µM), ATR inhibitor NU6027 (10 µM), DNA-PK inhibitor NU7441 (1 µM). After 2 hours of incubation, mitotic wash off was carried out and mitotic cells were re-plated in the media containing nocodazole/BI2536 for further 2 hours. Cells were collected and proceeded for immune-staining. These results are average of 3 independent experiments. The numbers on the bars refer to p-value as determined by t-test.

4.3.7 Aurora B kinase activity is important in DNA damage response observed by PLK1 inhibition

Spindle assembly checkpoint is responsible for monitoring the phases of mitosis. SAC prevents anaphase onset by targeting the anaphase promoting complex/cyclosome (APC/C, an E3 ubiquitin ligase). APC targets cell cycle proteins for degradation by 26S proteasome and by that it triggers the transition from metaphase to anaphase (Acquaviva and Pines, 2006). Aurora B is a kinase involved in SAC signalling. It is involved in error correction of microtubule-kinetochore attachment and, as a result, correct chromosome segregation (Kelly and Funabiki, 2009).

It has been reported that telomeric DNA damage induced by microtubule poison, Taxol, is dependent on Aurora B (Hayashi *et al.*, 2012). We were therefore interested to investigate whether the DNA damage induced by PLK1 inhibition is dependent on Aurora B kinase activity too. An inhibitor of Aurora B (ZM 447439) was used to test if Aurora B is involved in the DNA damage caused by prolonged mitosis. Aurora B was found to be partially responsible for the DNA damage response observed by both nocodazole and BI2536 as the number of γ-H2AX foci reduced in cells pre-treated with Aurora B inhibitor (**Figure 4-10**).

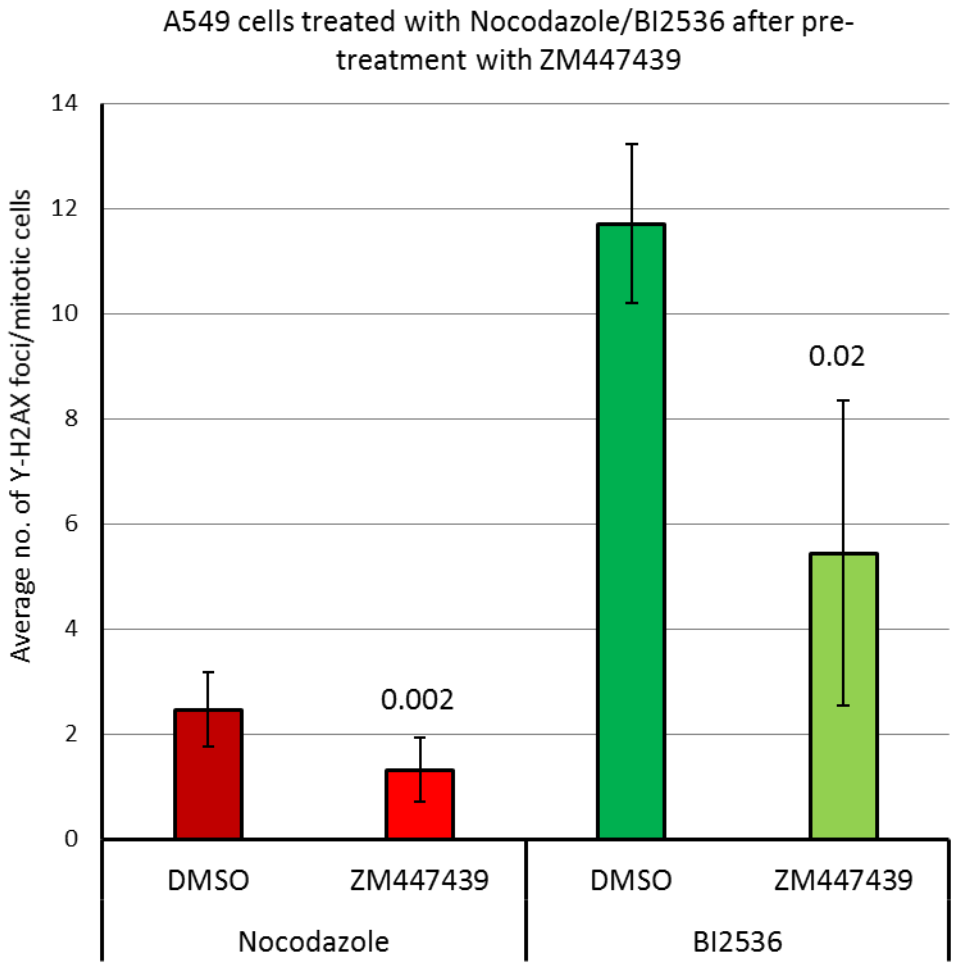


Figure 4-10. Aurora kinase B is involved in DNA damage response induced by PLK1 inhibition. A549 cells treated with 10 nM BI2536 or 100 ng/ml nocodazole with or without 30 minutes pre-treatment with Aurora B inhibitor (ZM447439). After 2 hours of incubation, mitotic wash off was performed and mitotic cells were re-plated in the media containing drugs and incubated for further 2 hours. Cells were then collected and proceeded for immuno-fluorescence. These results show the average of 7 independent experiments. The numbers on the bars refer to p-values as determined by t-test.

4.3.8 PLK1 inhibition could cause re-activation of DNA repair in mitosis

Mitotic cells and interphase cells respond differently to DNA damage. In interphase cells, DNA damage causes a halt in the cell cycle progression followed by recruitment of DNA repair factors to maintain genome integrity. On the other hand, in mitotic cells, a primary DNA damage response is activated (detection and marking the damage) which does not proceed to cell cycle arrest unless the damage is very severe. Also, DNA repair is shut down during mitosis and repair is delayed until cells exit mitosis. This reprogramming of DDR signalling shows that cells prioritise mitotic progression over activation of full DDR (signifying the importance of short mitosis) (Giunta *et al.*, 2010; Heijink *et al.*, 2013).

53BP1 one of the components of DNA damage repair pathway is phosphorylated by CDK1 and PLK1 in mitosis. Priming phosphorylation of 53BP1 by CDK1 generates a docking site for PLK1. PLK1 can then phosphorylate 53BP1, as a result modified 53BP1 cannot bind to the chromatin. This could be the reason why 53BP1 cannot localise to the site of DNA damage during mitosis (Benada *et al.*, 2015; Orthwein *et al.*, 2014) (**Figure 4-11**). This raised the possibility that inhibition of PLK1, and thus inhibition of 53BP1 phosphorylation by PLK1, could re-activate DNA repair in mitosis.

To investigate this, we used the PLK1 inhibitor, BI2536, to treat A549 cells. Immuno-fluorescence studies were performed on fixed cells using an antibody against 53BP1 and number of 53BP1 foci in mitotic cells were counted. As results demonstrate (**Figure 4-12 A**), BI2536 treated mitotic cells show more 53BP1 foci as compared with normal mitotic cells and even compared to nocodazole treated cells. Surprisingly when stained with both 53BP1 and γ-

H2AX to check their co-localization, many 53BP1 foci were not co-localized with γ-H2AX (**Figure 4-12 B**).

Results were further confirmed by using U2OS GFP-53BP1 cells which express doxycycline-inducible GFP-53BP1. These cells were generated in our laboratory by Dr. Rutschow. Immuno-fluorescence studies were performed on fixed cells and number of 53BP1 foci were counted (using GFP signal). Here again similar results were obtained; BI2536 treated mitotic cells showed 53BP1 foci more than normal mitotic cells and even more than nocodazole treated cells (**Figure 4-12 C).**

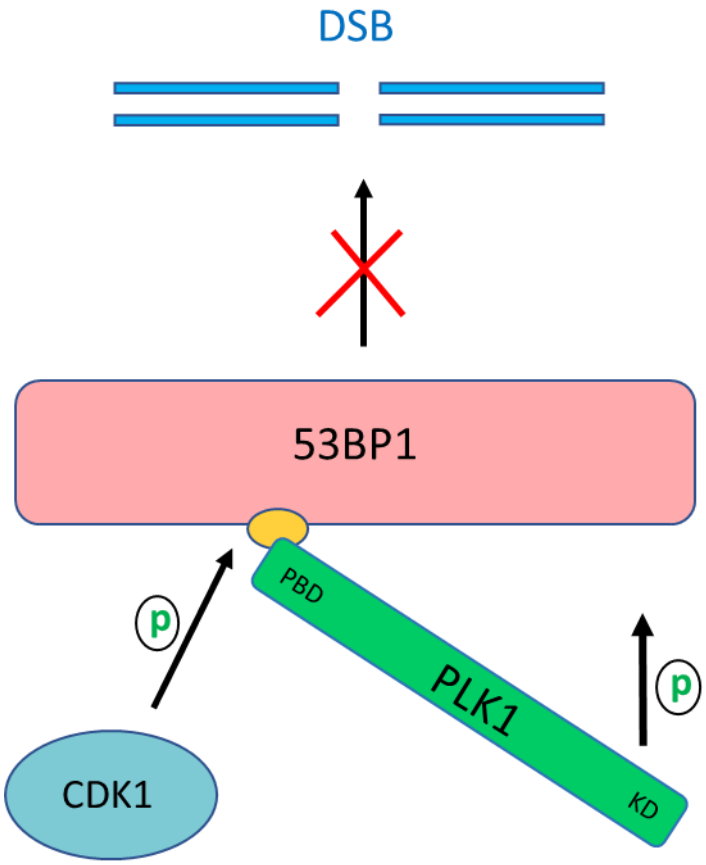
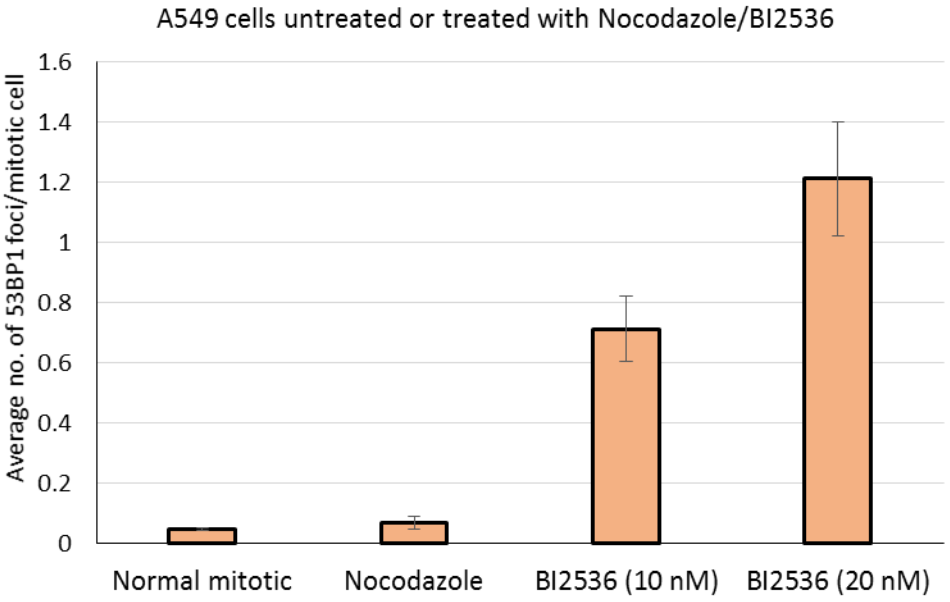


Figure 4-11. Schematic showing inhibition of 53BP1 recruitment to the site of DNA damage in mitosis. 53BP1 is phosphorylated by PLK1 and CDK1 in mitosis. CDK1 phosphorylates 53BP1 and generates a docking site for PLK1. Phosphorylation of 53BP1 by PLK1 inhibits its recruitment to the DNA damage site in mitosis.

A.



B.

Treatment	% of 53BP1 foci co-localised with γ-H2AX
Nocodazole	33.33
BI2536 (10 nM)	66.7
BI2536 (20 nM)	78.6

C.

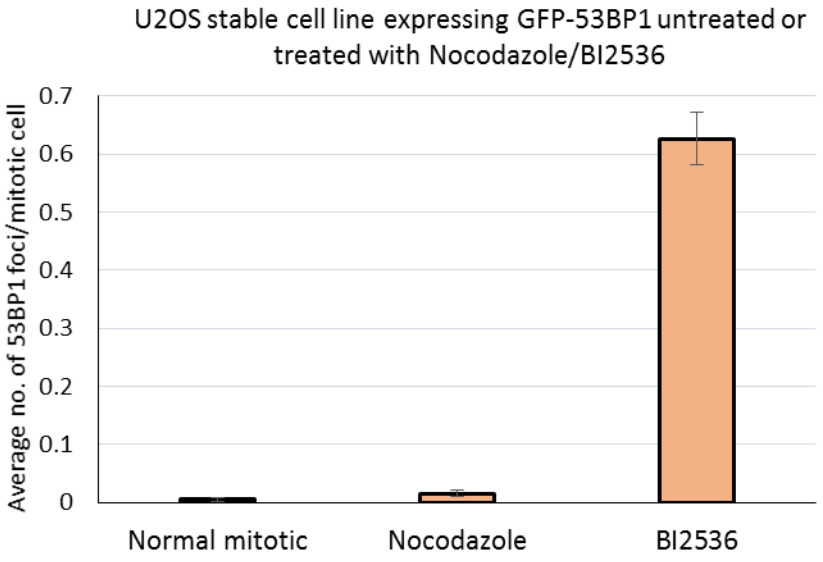


Figure 4-12. Re-activation of DNA repair in mitosis by inhibition of PLK1.
(A) A549 cells were treated with nocodazole or BI2536 for 2 hours. Floating cells were then collected and incubated for another 2 hours in the same media. Cells were then collected, cytospun and immuno-stained with anti-53BP1 and γ-H2AX antibody. **(B)** Co-localisation of anti-53BP1 and γ-H2AX foci in the samples in figure A were measured. **(C)** U2OS cells expressing GFP-53BP1 were treated with 100 ng/ml nocodazole or 25 nM BI2536 and incubated for 2 hours. Floating cells were then collected and incubated for another 4 hours in media containing nocodazole/BI2536. Cells were then collected, cytospun and proceeded for immune-fluorescence. These results show the average of at least 2 independent experiments.

4.3.9 Cells treated with BI2536 show more surviving colonies as compared with nocodazole treated cells

We were then interested to see the long-term effects of mitotic stress on cell survival and proliferation. A549 and U2OS cells were seeded and treated with nocodazole or BI2536. Mitotic wash off was carried out after 2 hours and collected mitotic cells were re-seeded in the media containing drug and incubated for further 2 or 4 hours (A549 or U2OS). Mitotic cells were then collected and washed twice to ensure no residual drugs have left. Cells were then counted and seeded in dishes containing fresh media. Normal mitotic cells were counted and seeded as the control for the experiment. Clonogenic survival assays were performed when colonies reached desirable size.

Analysis of the results showed that the number of surviving colonies is greater when cells are treated with BI2536 compared to nocodazole (**Figure 4-13**). These findings are particularly interesting because if we look back at the results we obtained in previous experiments shown in Figure 4-6, the number of γ-H2AX foci in cells treated with BI2536 is higher compared to nocodazole treated cells so it was expected to see less number of surviving colonies in BI2536 treated cells compared to nocodazole treated cells.

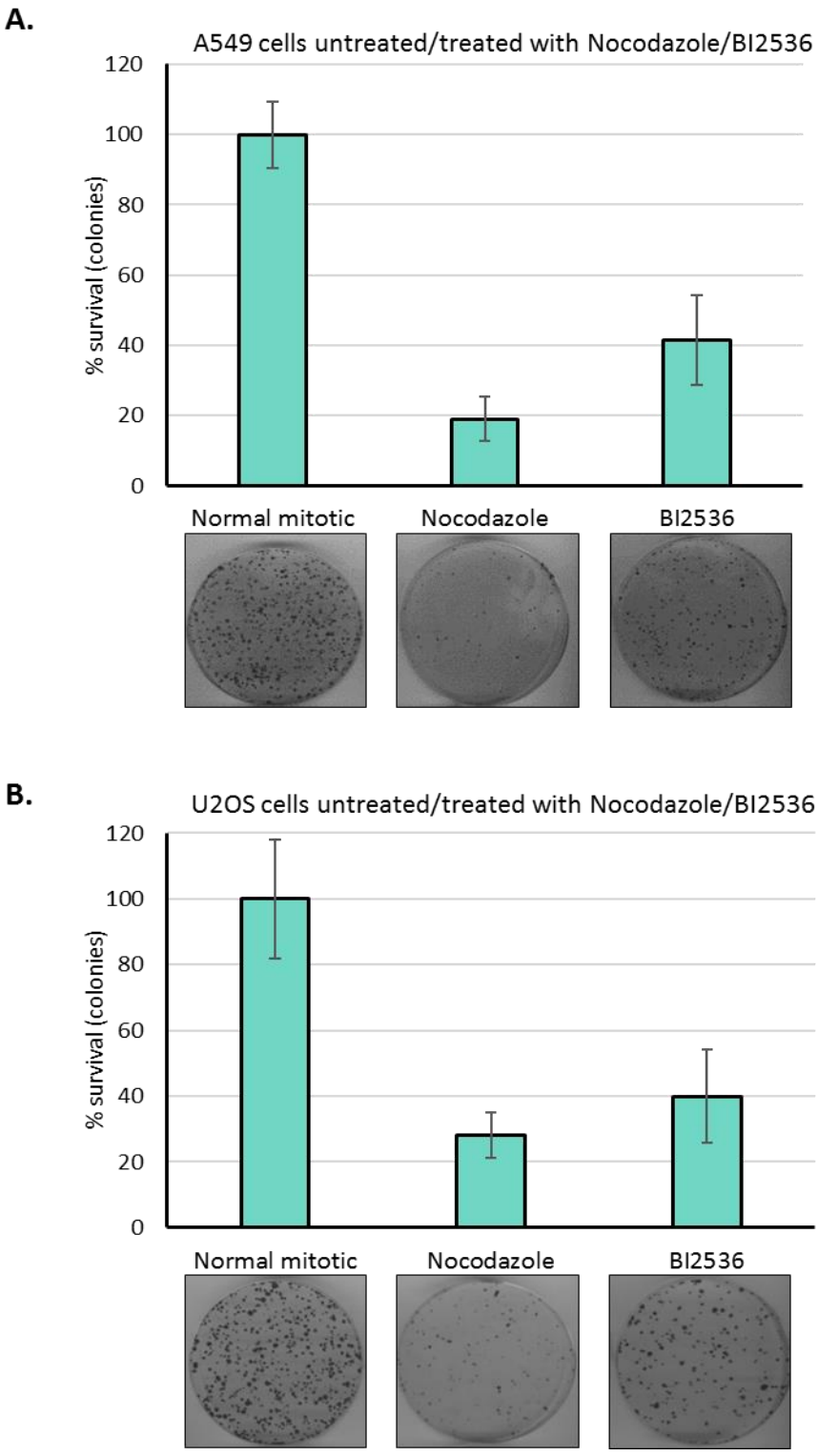


Figure 4-13. Cells treated with BI2536 show more surviving colonies compare to nocodazole treated cells.

(A) A549 cells were treated with 100 ng/ml nocodazole or 10 nM BI2536 for 2 hours. Mitotic wash off was then carried out and mitotic cells re-plated in the media containing drugs for further 2 hours. **(B)** U2OS cells treated with 100 ng/ml nocodazole or 25 nM BI2536 for 2 hours. Mitotic wash off was then carried out and mitotic cells re-plated in the media containing drug for further 4 hours. Cells were then counted, and 1500 cells were added to each 10 cm dish containing fresh media. Following 9-10 days incubation number of survived colonies were counted in each dish. The figure shows average of 3 experiments and representative images. These results are average/representative (graph/images respectively) of at least 2 independent experiments.

4.4 Discussion

The DNA damage after prolonged mitosis and the underlying mechanisms remain interesting areas of research to be investigated. Some mechanisms have been proposed for this, such as partial activation of the apoptotic pathway which causes partial activation of CAD and limited DNA damage (Orth *et al.*, 2012) but still more research needs to be done to fully understand it.

We have shown in this chapter that PLK1 inhibition arrests the cells in mitosis and we have tried to use this to widen our understanding of mitotic regulation and DNA damage after prolonged mitosis and the consequences of this inhibition. As our group and most of the published literature have previously worked on the mitotic arrest induced by microtubule poisons, parts of this chapter compared how PLK1 inhibition-induced mitotic arrest and the consequences associated with such arrest are different from that induced by microtubule poisons.

In agreement with published literature, treatment with a PLK1 inhibitor resulted in mitotic arrest and consequent DNA damage in our experiments (Lénárt *et al.*, 2007; Steegmaier *et al.*, 2007; Driscoll *et al.*, 2014). We also used nocodazole and confirmed the mitotic arrest and resulting DNA damage that have been reported with microtubule poisons before (Dalton et al. 2007; Hain *et al.*, 2016; Orth *et al.*, 2012). However, our studies indicated a different DNA damage response mechanism between microtubule poisons and the PLK1 inhibitor.

Several lines of evidence have shown the initiation of a DNA damage response following prolonged mitosis by microtubule poisons; however the damage had been reported to be mainly on telomeres and was suggested to be caspase dependent (Hain *et al.*, 2016, Orth *et al.*, 2012, Hayashi *et al.*, 2012) . Findings

of this chapter suggest that the PLK1 inhibition causes formation of γ-H2AX foci equally at the telomeric and non-telomeric regions. Also, we found that the DNA damage induced by PLK1 inhibition is not caspase or CAD dependent as using a pan caspase inhibitor did not significantly reduced the number of γ-H2AX foci, neither did the use of ICAD mutant cells.

TRF2 loss was investigated and it seems to be greater following nocodazole treatment compared to BI2536 treatment. This observation could be true since nocodazole results mainly in telomeric foci, whereas the ratio of telomeric and non-telomeric foci in BI2536 treated cells are equal. However, drawing any conclusions from this experiment requires further experimentations as the experiment has not been done in replicates due to shortage of time. Also, it would have been helpful to count the γ-H2AX and TRF2 foci in the same experiment in order to eliminate the possibility that the higher loss of TRF2 observed by nocodazole is due to higher DNA damage.

In nocodazole-induced mitotic arrest, DNA-PK was found to be the main kinase involved in the mitotic DNA damage response, which is consistent with previous report (Hain *et al.*, 2016). However, in mitotic arrest caused by PLK1 inhibition, we found that both ATR and DNA-PK are involved. Combining DNA damage sensing kinase inhibitors together would be a helpful approach to determine the significance of these kinases in the DNA damage response observed and investigate if there might be redundant pathways involved.

Another interesting concept investigated in this chapter, is the fact that using a PLK1 inhibitor results in formation of 53BP1 foci, indicating that PLK1 inhibition might activate DNA-repair in mitosis. Interestingly, 53BP1 foci are not all localised at γ-H2AX foci. What would be the consequences of recruitment of

53BP1 and activation of DNA repair in mitosis? It would be an interesting area of research to be followed. Clonogenic assays indicate that BI2536 treated cells form more colonies compared to nocodazole treated ones. This is particularly interesting because when looking at the number of γ-H2AX foci, the numbers are higher with PLK1 inhibitor compared to nocodazole. Could it be related to reactivation of DNA repair in mitosis by PLK1 inhibitor? Or does it mean that PLK1 inhibited cells undergo repair more than nocodazole treated cells? This remains to be investigated. More clonogenic assay investigations for cells treated with PLK1 inhibitor with pre-treatment with kinase inhibitors could give us an indication of the effect of combination of these agents on survival of cancer cells in long term.

Overall, the data in this chapter add on the previously published literature about mitotic arrest-induced DNA damage response and demonstrate some differences in the DNA damage response mechanisms observed by PLK1 inhibitor and nocodazole. The exact cause of the damage is unknown. Further work could attempt to address this question and also what would be the consequences of PLK1 inhibition in terms of cell fate.

Chapter 5: Conclusions and Future Perspectives

Given the importance of PLK1 in oncogenesis and its many roles in cell cycle, especially during mitosis, research into regulation of this protein is an important area for investigations. In this thesis, we have tried to address two of the key questions in PLK1 research and what we have concluded is discussed below.

Investigating PLK1 as a target of p53 transcriptional regulation, was one of the issues that tried to be addressed in this thesis. The main focus was in light of the recent model (p53-p21-DREAM-CDE/CHR) proposed by Fischer, Quaas, Nickel, *et al.* (2015). While trying to reproduce the data that show p53 represses PLK1, different p53-inducing agents seemed to give rise to completely different changes in the PLK1 levels. Nutlin, a pharmacological inhibitor of MDM2, caused significant depletion of PLK1, whereas an agent like etoposide appear to stimulate PLK1 levels. Cell cycle analysis revealed that different drugs lead to different cell cycle profile and therefore we concluded that the apparent regulation of PLK1 by p53 might be a reflection of cell cycle periodicity.

Whether p53 represses *PLK1* through direct or indirect mechanisms and exactly which elements/molecules are involved, remained unresolved. The data in this thesis support a cell line dependency for the mechanism(s) by which this phenomenon occurs. The conflicting data published by different groups could indicate the possibility of multiple mechanisms. Consistent with that hypothesis, our findings showed that in U2OS cells CDE/CHR elements are only partly responsible for *PLK1* repression and there should be other mechanism(s) involved. **Figure 5-1** depicts our proposed model on potential mechanisms p53 may recruit to downregulate PLK1.

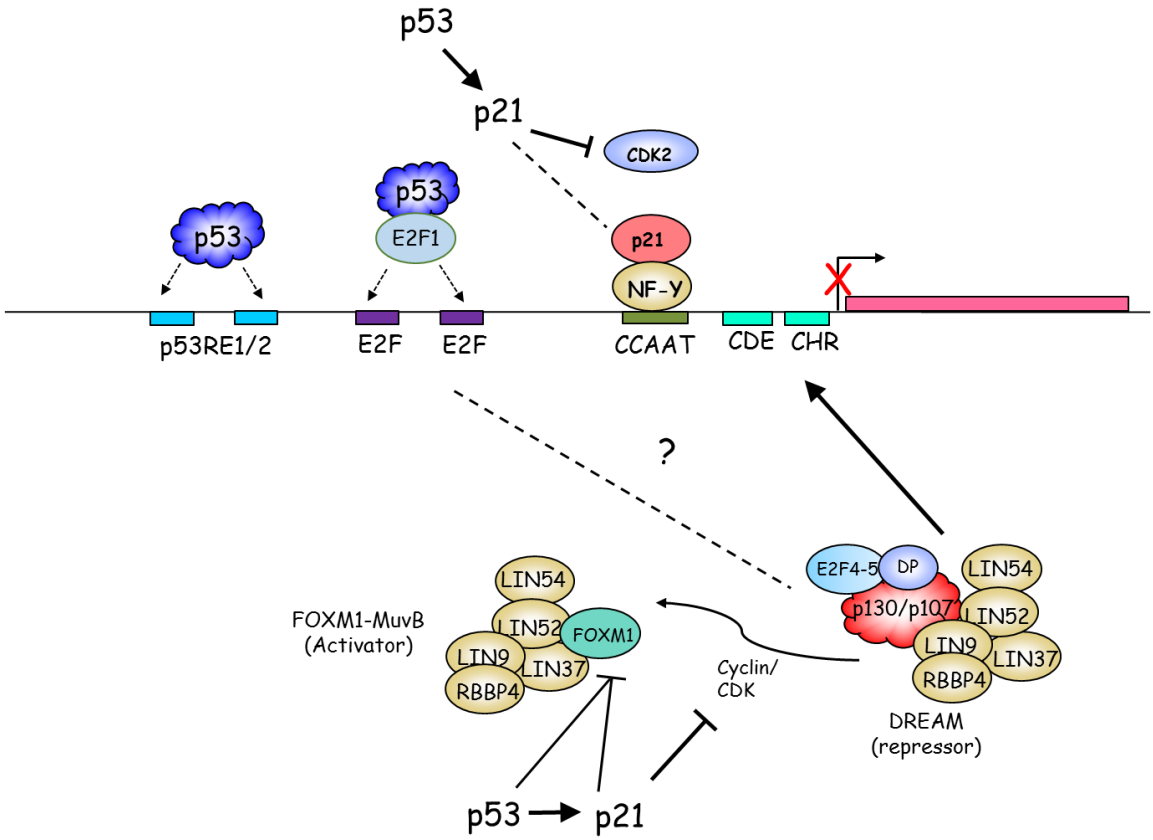


Figure 5-1. Schematic representation of possible p53 mediated *PLK1* repression mechanisms.
Various proposed p53 mediated mechanisms for PLK1 repression, has been depicted. P53 can repress *PLK1* by direct interaction with the promoter at p53REs. p53 has also been reported to form an inhibitory p53-E2F1-DNA complex and inhibit E2F1 mediated expression of PLK1. p53 has been suggested to mediate *PLK1* repression through its downstream target p21 with several different mechanisms. One mechanism involves p21 mediated inhibition of cyclin/CDK, this keeps p130/p107 in hypo phosphorylated state and hence stabilisation of DREAM complex. Binding of DREAM complex to CDE/CHR mediates *PLK1* repression. p53 induced p21 can also replace CDK2 in interacting with NF-YA and cause CCAAT box to have repressive function. p53 can also inhibit FOX M1, which acts in a positive feedback loop with PLK1. Could DREAM complex associate with the E2F binding sites in the *PLK1* promoter (like G1/S genes)? That could be another possibility which has not been reported/investigated.

What would be the consequences of p53 mediated repression of *PLK1* in long term and how it can affect the cell fate? Further work could attempt to address this key question. PLK1 is a central protein during mitosis and many mitotic events depend on PLK1 activity. If p53 mediated repression of *PLK1* (as a result of treatment with Nutlin or DNA damaging agents) arrests the cells and/or prevents them from entry into mitosis, it would be interesting to know what will happen in long term? Would *PLK1* repression recover and would cells continue to cycle? Or long-term arrest triggers the apoptotic signals?

Since p53 not only represses *PLK1*, but many other G2/M cell cycle genes in response to DNA damage, understanding the mechanism(s) of repression of PLK1 by p53 could potentially apply to other genes involved in G2/M DNA damage checkpoint. In fact, it would be interesting to see if the same mechanism(s) can apply to other G2/M expressing genes that are p53 targets. p53-mediated regulation of G2/M genes is important as it causes G2/M arrest in response to DNA damage. This arrest provides time for repair of the damage or an opportunity to permanently arrest severely damaged cells, both of which are important to protect organisms against accumulation of aberrant cells (Taylor and Stark, 2001).

Since PLK1 also inhibits the function of p53 (as discussed in section 1.2.7), PLK1 downregulation is important not only because it halts the cell cycle progression but for maintaining p53 activity and functions during stress conditions. Downregulation of PLK1 could mean that p53 can recruit to the target genes without disruption by PLK1.

As PLK1 has a central role during different events of mitosis, and because it is required for the progression of the cell cycle, further investigations of how this

protein is regulated could potentially lead to better combination therapies or could eventually lead to developing new therapies.

Trying to develop novel therapies that target specific proteins with differential expression in normal and cancer cells is one of the important approaches in cancer therapy. PLK1 is one such protein and hence a potential target in cancer treatment. Therefore, part of this thesis investigated the **mitotic arrest and resultant DNA damage response induced by PLK1 inhibition**. Consistent with other studies (Lénárt *et al.*, 2007; Steegmaier *et al.*, 2007; Driscoll *et al.*, 2014), our results showed that inhibiting PLK1 results in mitotic arrest and formation of γ-H2AX foci, an indicator of DNA damage. However, in addition to this, the data in this thesis showed that the resulting damage occur equally at both telomeric and non-telomeric regions and was found to be independent of caspases. These data are different from the mitotic arrest induced DNA damage resulting from treatment with microtubule poisons which was reported to be mostly telomeric and caspase dependent (Hain *et al.*, 2016; Orth *et al.*, 2012; Hayashi *et al.*, 2012). The caspase-independency of the DNA damage induced by PLK1 inhibition has been recently reported using a different approach (Smith *et al.*, 2017). Thus, the data in this thesis add to the previous published literature by demonstrating some differences in the mitotic arrest-induced DNA damage response observed by PLK1 inhibition and microtubule poisons.

One of the very interesting concepts investigated in this thesis, is the fact that PLK1 inhibitor re-recruits DNA repair factor 53BP1 in mitosis. Why 53BP1 foci are not all localised at γ-H2AX foci is unclear. Anyhow, the recruitment of DNA repair factors by PLK1 inhibition in mitosis is not beneficial. Firstly, because

DNA repair activation in mitosis prolongs the period of stressful mitosis which could be catastrophic and potentially lead to acquiring even more damage. Also, repair requires disruption of the highly compacted chromosome structure, probably causing various segregation defects (Giunta and Jackson, 2011). Additionally, homologous recombination (HR) does not happen during mitosis due to condensed chromatin. The only error correction machinery which may occur in mitosis is non-homologous end joining (NHEJ) which is not error free. Thus, DNA repair activation in mitosis by NHEJ can cause translocation of chromosomes and telomere fusion which is very harmful and can cause aneuploidy (Benada *et al.*, 2015). This finding highlights the importance of further investigating the chromosomal abnormalities in cells that have undergone treatment with PLK1 inhibitor. This finding also suggests combination of PLK1 inhibitors and drugs that block NHEJ.

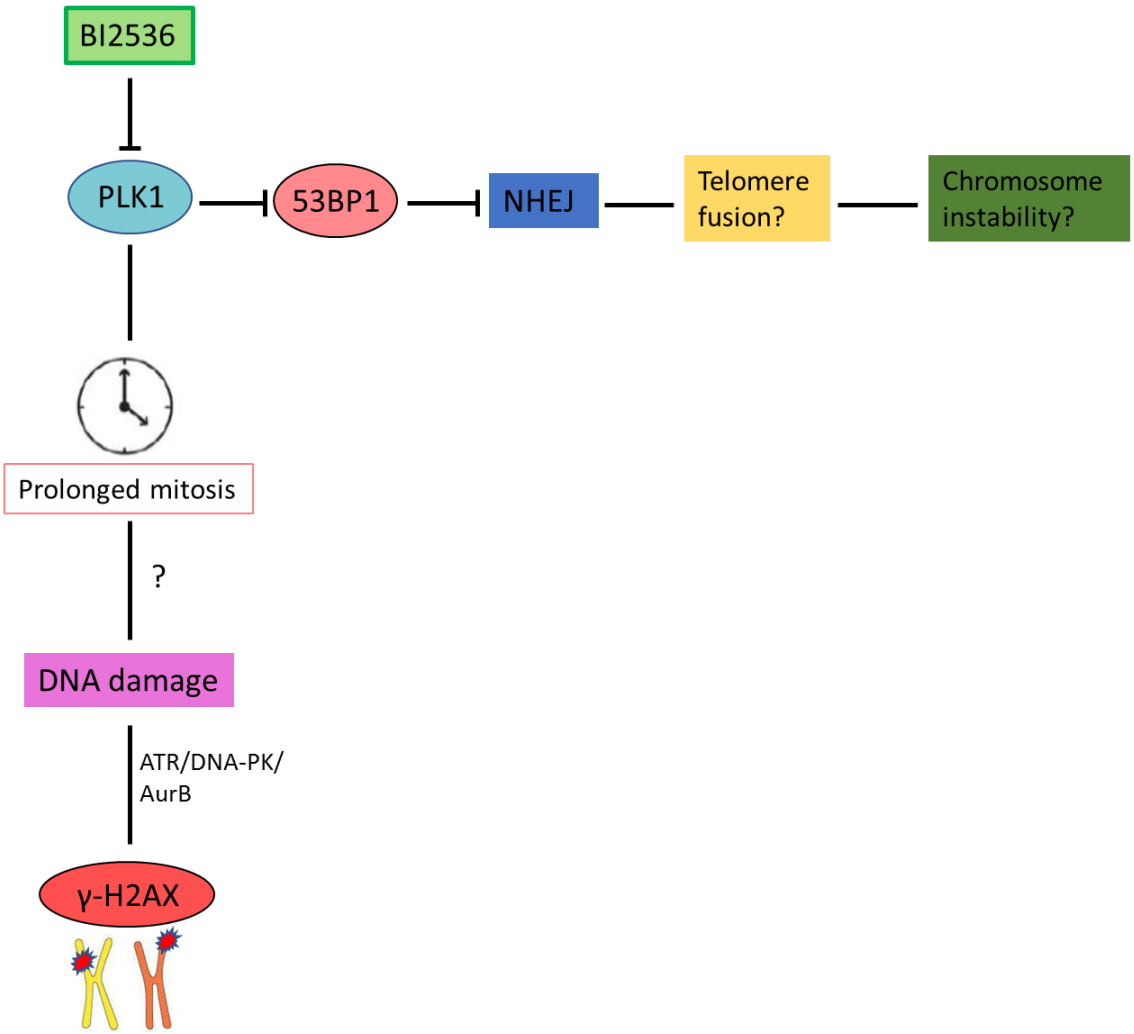


Figure 5-2. Schematic representation of events occurring after PLK1 inhibition in mitosis.
PLK1 inhibition results in prolonged mitosis and resultant DNA damage response. This DNA damage was found to be both telomeric and non-telomeric and was attenuated by inhibition of ATR/DNA-PK/Aurora B. PLK1 inhibition causes recruitment of 53BP1 to the site of damage and may activate DNA repair in mitosis which could be harmful as it can cause telomere fusion and chromosomal instability.

In this thesis we have performed experiments to investigate the level of DNA damage in mitotically arrested cells. We still don't know what the consequences of PLk1 inhibition would be when cells exit mitosis either by releasing them from drug contained media or if they manage to escape/slip from mitosis. Investigating these would be beneficial as it could give us information about how PLK1 inhibition affects cell fate.

Understanding the mechanisms PLK1 inhibitors follow and the cross talk between PLK1 and other proteins could guide clinicians when choosing PLK1 inhibitors for patients with specific tumour genotypes. For example, many studies have shown that p53 status of the cancer cells could determine the level of effectiveness of PLK1 inhibitors, with p53 being a disadvantage in PLK1 inhibition therapy (Smith *et al.*, 2017; McKenzie *et al.*, 2010; Degenhardt *et al.*, 2010). On the other hand, previous investigations reported that the sensitivity of different cancer cells to Taxol are p53 independent (Debernardis *et al.*, 1997; Reinecke *et al.*, 2005). Also, in p53 depleted U2OS cells, after mitotic arrest with nocodazole, there has been no accumulation of cells in G1 but more apoptosis compared to normal U2OS cells (Colin *et al.* 2015). Since both microtubule poisons and PLK1 inhibitors cause arrest in mitosis, it is interesting that the presence of p53 is advantageous/ineffective in one therapy and adverse in another.

In our studies we used cell lines with wild type p53. As it has been shown that p53 is a disadvantage for therapy when treating the cells with PLK1 inhibitor, further investigations would be helpful to determine the differences in the action of microtubule poisons and PLK1 inhibitors in p53 null cells. For example, with PLK1 inhibitors we observed more damage, but less surviving colonies

compared to nocodazole, it would be interesting to see if we get the same results in cells with p53 null background.

The cause of the DNA damage-induced PLK1 inhibition remains to be investigated. It is unclear if the DNA damage observed is directly due to mitotic arrest or due to the activation of SAC. Understanding these could help in developing new strategies for cancer treatment. If the DNA damage trigger is mitotic arrest, then prolonging the arrest by means other than SAC activation (for example knockdown of activator of APC/C, CDC20) would be beneficial. Whereas if trigger is SAC activity, trying to hyper activate SAC (for example by partial inhibition of kinetochore protein) could result in improved therapeutic mechanisms.

It will be interesting to characterise the pathway PLK1 inhibition follows in mitotic cells in the context of PLK1 as a potential drug target. Understanding these mechanisms can help to improve existing therapies and potentially can help in developing new agents to treat cancer. Also knowing these mechanisms is useful when considering combination therapies.

Finally, from understanding the biology of PLK1 to its clinical application, there is probably a long way to go. Many fundamental questions are still waiting to be answered such as: the true relationship between PLK1 and p53 (Liu *et al.,* 2017), whether PLK1 overexpression in cancers is a cause or consequence of cancer (Cholewa *et al*., 2013)? The key interactive network of PLK1 underlying the carcinogenesis (Liu, Sun and Wang, 2017) and how exactly PLK1 inhibition can help in cancer therapy. Research into understanding PLK1 itself and its regulators/inhibitors, would be advantageous not only in cancer but also in

understanding the pathogenesis of other diseases that involve PLK1 deregulation and could potentially lead to discovering therapies for them.

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